**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of:
Riqiang Yan *et al.*

Application Nos.:; 10/801,938; 10/801,493; 11/713,091 and 11/753,331

Art Unit: 1639

For: SUBSTRATES AND ASSAYS FOR *B*-SECRETASE ACTIVITY

Examiner: J.S. Lundgren

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

**DECLARATION OF JOHN P. ANDERSON, Ph.D. UNDER 37 CFR 1.132**

I, John P. Anderson, Ph.D., hereby declare as follows:

**A. Declarant's Background, Qualifications and Disclosure of Interest**

A1. The patent applications identified above concern β-secretase enzyme substrates and assays that employ such substrates. I have extensive experience and education in biochemistry and the experimental methods and analyses described in the patent applications. My *curriculum vitae* provides evidence of my experience in the field and is included as Exhibit A.

A2. The U.S. patent applications identified above are owned by Elan Pharmaceuticals, Inc. I am currently employed by Elan Pharmaceuticals, Inc.

**B. Purpose of the Declaration**

B1. The patent applications identified above, U.S. Patent Application Nos. 10/801,938, 10/801,493, 11/713,091 and, 11/753,331 share a common specification (description of the invention) and share the same original filing dates of July 19, 2000 (Provisional U.S. Patent Application No. 60/219,795), March 12, 2001 (Provisional U.S. Patent Application No. 60/275,251) and July 19, 2001 (U.S. Patent Application No. 09/908,943). I have reviewed the patent specification in detail. Briefly, the specification describes β-secretase (BACE) enzymes, non-naturally occurring substrates of these enzymes and uses of such substrates (*e.g.*, use in assays to identify inhibitors of β-secretase enzyme activity). I have also reviewed the claims that are currently pending in each of the patent

applications identified above. Thus, I am familiar with the disclosure of the invention provided by the specification and the claims presented by the inventors based upon the disclosure. The currently pending claims for each of the cases are appended as Exhibits B, C, D and E as indicated below.

B2. I understand that in each of the identified patent applications, the pending claims have been rejected by the U.S. Patent and Trademark Office. I have reviewed the current rejections of the claims in each of the cases and the points raised by the Examiner are nearly identical with respect to each of the applications. For simplicity I will refer to the Final rejection in U.S. Patent Application No. 10/801,938 that is dated September 15, 2009 even though, unless stated otherwise, my comments are applicable to all of the applications listed above.

B3. I understand that the Examiner has rejected claims under the "written description" section of the patent laws and taken the position that a skilled artisan would not have recognized that the inventors were in possession of the invention, as presently claimed, at the time the application was filed. In particular, the Examiner states that "[t]he claims contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention," Action at page 2.

B4. The written description requirement has been explained to me and I understand that an essential goal of the description of the invention requirement is to clearly convey that an applicant has invented the subject matter which the applicant claims as the invention. I understand that another objective of this requirement is to put the public in possession of the invention that is claimed. To satisfy the written description requirement, a patent specification must describe the claimed invention in sufficient detail that a person of average skill in the field of the invention can reasonably conclude that the inventor(s) had possession of the claimed invention. An applicant shows possession of the claimed invention by describing in the patent application the claimed invention with all of the details that are found in the claims using descriptive means such as words, structures, figures, diagrams, tables and formulas that fully set forth the claimed invention. There is no requirement that the language of the claims be found word-for-word in the application, as long as the subject

matter that is claimed is supported in the specification as a whole, through some combination of express, implicit or inherent disclosure.

B5. The process of determining the adequacy of an application's written description involves interpreting the claims, to determine what the claims cover; and reviewing the application in its entirety to understand whether and how the application provides description of the subject matter covered by the claims. The subject matter of a claim would be the structure of a product defined by a claim, or the acts of a process defined by a claim, for example. This review is conducted from the standpoint of a person of average skill in the field of the invention at the time that the application was filed, with the understanding that the application is written for such persons and the specificity of the disclosure need not be as detailed with respect to features within the knowledge and skill of such persons. Thus, information which is well known to those in the field of the invention need not be described in detail in the application.

## C. General Observations

### (i) Introduction and Background

C1. The subject matter of the applications has been partly summarized by the Examiner in the paragraphs bridging pages 4 and 5 of the Final Office Action. The Examiner indicates that one aspect of the invention concerns methods for screening for inhibitors of a class of aspartyl protease enzymes, known as β-secretase (or BACE) enzymes, that are involved in the progression of Alzheimer's disease (AD). Briefly, the Examiner states:

> Applicants provide a clear and succinct background of the invention by detailing certain biochemical pathways in the formation of plaques responsible for AD. An origin of these plaques is the amyloid protein precursor (APP), which when first processed by an enzyme having β-secretase activity, followed by an enzyme having γ-secretase activity, causes the formation of a 40/42 amino acid peptide plaque known as Aβ.
>
> Accordingly, the development of methods for identifying compounds that might one day serve as potential β-secretase inhibitors are undoubtedly needed by the biomedical community in order to accelerate the development of AD drug candidates. Action at pages 4-5.

For the purposes of such laboratory methods, the wild type (wt) human APP polypeptide is cleaved by β-secretase inefficiently *in vitro* and preferred substrates of the invention are

substrates that are cleaved more efficiently than wt-APP. Even more preferable are substrates that are cleaved more efficiently than the 'Swedish mutant' of APP that is known to be cleaved very efficiently and is associated with an early onset inherited form of AD in a small Swedish population (*see, e.g.,* page 6 of the specification).

C2. A scientist with typical experience in the technical field of the applications around the years of 2000 or 2001 would have had a Ph.D. in molecular biology, biochemistry or a related discipline and at least a few years of post-doctoral research experience. Based on my educational background and research experience, I would have been considered a person of at least ordinary skill at the time the applications were originally filed. My *curriculum vitae* provides evidence of my experience and education in this technical field and is included as Exhibit A. Based on my substantial expertise I consider myself qualified to render an opinion about what "a person of ordinary skill in the art" would have understood regarding the invention described the patent applications at the time that they were filed. Unless specifically stated, all of the observations and opinions set forth below reflect what a person of average skill would have concluded at the time the applications were filed *e.g.,* around July 19, 2000.

**(ii) Teaching of the Various Classes of the Invention**

C3. A review of the applications reveals a detailed description of β-secretase substrates and methods for their use. Pages 3-6 of the specification detail some of the general, physical characteristics of β-secretase substrate peptides. In general, the substrates are defined by the amino acids that occupy certain positions relative the β-secretase cleavage site. The positions are numbered using a nomenclature that was standard in the field. The positions on the amino-terminal side of the cleavage are indicated with a "P" and a subscripted number indicating the distance from the cleavage site. Likewise, positions on the carboxyl-terminal side of the cleavage are indicated in an identical fashion with the addition of a 'prime' notation above the Arabic numeral. For example, the four amino acids surrounding the β-secretase cleavage site are designated $P_2$ $P_1$ $P_{1'}$ $P_{2'}$ and are amino acids "KMDA" in wild type APP protein, and amino acids "NLDA" in the Swedish mutation form of APP (using standard one-letter abbreviations for the amino acids). On pages 3 and 4, for example, the application discloses that a substrate will comprise at least 4 amino acids at positions $P_2$, $P_1$, $P_{1'}$ and $P_{2'}$ relative to the cleavage site (*i.e.,* wherein the cleavage site is

between $P_1$ and $P_{1'}$). The specification goes on to describe the physical properties that are preferred at each of the four positions that flank the protease cleavage site as well as positions that are more distant from the site of cleavage. In addition to basic peptide substrates and fusion proteins comprising the peptide sequences, a number of additional modified substrates are disclosed in the patent applications. For example, peptides may comprise tags or labels. In particular, a number of internally quenched labels are described (see, the paragraph spanning pages 37-38). Fusion proteins and chimeric proteins that may be constructed from the substrates are also described on pages 7 and 8. For instance, on page 8, lines 4-8, APP derivative molecules are discussed wherein the β-secretase cleavage site is modified to match a disclosed substrate sequence. In further aspects, polynucleotide constructs encoding substrates are described as well as transformed cells and animals for expression of substrates sequences. Uses for substrates of the invention include assay methods for identifying modulators of β-secretase activity. For example, on page 9, lines 1-8, methods for assaying for modulators of β-secretase activity using a substrate and a mammalian β-secretase are described. Assay methods may further involve measuring the cleavage of a substrate in presence or absence of a putative modulator to identify a modulator of β-secretase activity (page 9, lines 7-13). Assay methods include cell free and cell based assays and a detailed description of specific assays is set forth in the Examples that begin at page 79.

**(iii) Teaching Regarding Peptide Substrates of the Invention**

**(a) Substrate Peptide Length**

C4. The claims that are currently pending in the various applications specify that the peptide β-secretase substrates have at least six amino acids. The specification describes peptide substrates that have, for instance, 6 or more amino acids (see, *e.g.*, page 27, lines 29-32) and the inventors specifically contemplated substrates comprising six or more amino acids on page 28, lines 1-3 as a peptide having a motif that comprises $P_3P_2P_1$-$P_{1'}P_{2'}P_{3'}$. The specification also teaches that longer peptides can be cleaved by β-secretase, and, in fact, longer peptides are preferred in that they can be cleaved more efficiently than shorter sequences. For instance, studies presented on page 25 and in Table 5 demonstrate that longer peptide substrates are cleaved more efficiently than otherwise identical, shorter peptides. The studies presented in the application tested peptide substrates that were 10 amino acids in length or larger. However, the disclosed studies were designed to determine the requisite

physical properties of residues that are proximal to the cleavage site and the length of the test substrates was maintained as a constant in this investigation. A person of ordinary skill would have understood that the study was not designed to demonstrate the minimum length of a suitable substrate. Thus, it would have been recognized that the invention encompassed substrates of at least four amino acids and that the inventors also specifically contemplated a genus of substrates having six or more amino acids.

**(b) Peptide Core Structure**

C5. The studies presented in the application elucidate the structure for novel synthetic β-secretase substrates by identifying preferred amino acid properties at the positions that define the β-secretase cleavage site, and in particular, the four residues that flank the cleavage site. It is these four residues that are most important to cleavage activity, accordingly the four cleavage cite proximal residues and their preferred physical properties are defined beginning at page 3, line 27, of the specification. The physical properties of a particular residue at a particular position is the most crucial factor that affects the ability of a substrate to be cleaved. Hence, by making single amino acid substitutions at a particular position while maintaining identical residues at other positions, the specific physical properties that are favorable to substrate cleavability at each position were elucidated by the inventors. Review of the studies comprising such substitutions at each position provides information regarding the general physical properties that are preferred and most preferred at each position. Based on these studies the inventors have deduced a genus of residues having similar physical properties that may be used at the given position to favor a cleavable substrate. Table 6 on page 30 of the application represents a summary of the specific amino acid residues that are preferred at the cleavage site proximal positions. It is clear to me and it would have been clear to a person of average skill in the field from Table 6 and related text that the inventors contemplated peptide substrates wherein $P_2$ is N, L, K, S, G, T, D, A, Q or E; $P_1$ is Y, L, M, Nle, F or H; $P_{1'}$ is E, A, D, M, Q, S or G; and $P_{2'}$ is V, A, N, T, L, F and S, each independently of the other. The reader understands Table 6 to provide a concise description of each of the 10x6x7x7=2,940 peptide substrates defined by independently selecting $P_2$ $P_1$ $P_{1'}$ and $P_{2'}$ from the choices provided in the Table. In addition to describing these 2,940 substrates individually and as a collective group, the application also describes

subsets (smaller groups) of more specific amino acids at certain positions that are shown to yield highly active substrates.

C6. The description of β-secretase substrates provided in the application includes data from a number of experimental studies. The inventors described studies of the β-secretase cleavage of decapeptide substrates including cleavage sites from ubiquitin and the oxidized insulin B chain. In general, the tested substrates was found to be cleaved less efficiently than a peptide corresponding to Swedish mutant form of APP; however, the oxidized insulin B chain substrate was found to be cleaved with similar efficiency (see, page 15, line 23 to page 16, line 15). The inventors then compared the initial test peptide sequences with sequences for cleavage sites in known or predicted β-secretase substrates (page 16, lines 12-15). From this analysis a second round of substrates was developed wherein amino acid substitutions were made in the background of an oxidized insulin B-chain sequence and tested for activity (*β*-secretase processing or cleavage). The second round of studies encompassed amino acid substitution at position $P_2$-$P_{3'}$ and the results of the studies are summarized in Table 2 (on page 20). Similarly, studies were conducted wherein amino acid substitutions were made at the $P_2$-$P_{3'}$ in a ubiquitin peptide background and it was found that each of the substrates was cleaved by *β*-secretase more efficiently than wild type APP and certain substrates were cleaved at a rate that approached that of the Swedish mutant peptide (*see, e.g.,* Table 3 on page 21 and the text spanning page 21, line 27 to page 22, line 4). Further studies examined the effect of amino acid substitution at positions $P_2$-$P_{2'}$ in an APP peptide background. These results are shown in the Table 5 on page 24. Taken together, the studies not only elucidate the preferred properties for amino acids in the four positions closest to the cleavage site, but also demonstrate that the cleavability of substrates is largely independent of the flanking peptide sequence. In other words, *β*-secretase substrates having a cleavage site defined by $P_2$-$P_{2'}$ can be cleaved regardless of whether they are flanked by sequence derived from APP, ubiquitin or the insulin B chain.

### (c) Amino Acids Flanking the Core Structure

C7. The Examiner asserts, at Pages 6-7 of the Office Action, that the application does not provide adequate teaching regarding the effect on the substrate cleavage efficiency of sequences flanking the four amino acids that are closest to the cleavage site. A person in the field would have expected from experience that the residues closest to the

cleavage site would have more influence on cleavage than more distant residues. Moreover, as summarized above in paragraph C6, when the four cleavage site proximal residues were moved from an ubiquitin to an insulin B-chain background, and then into a wild-type APP peptide background, cleavage activity was observed in all cases. Thus, experience with other enzymes and more importantly actual evidence described in the application for β-secretase indicated that residues more distant from the cleavage site typically have much less effect on cleavability of the substrate and that a wide variety of amino acid substitutions in the flanking sequence could be employed. In addition, the application provides extensive teaching regarding residues that are preferred at more distant amino acid positions. For example, Table 6 on page 30 presents the preferred amino acid substitutions from position $P_4$-$P_{4'}$ that were deduced by the inventors based upon the studies presented in the applications. Furthermore, as outlined above, results shown in Table 5 on page 25, demonstrate that additional of amino acid residues to the amino-terminus of a substrates can enhance cleavage efficiency (*i.e.*, compare the cleavage results with SEQ ID NO: 145 to SEQ ID NO: 141). The inventors provide exemplary larger proteins that can be used as a basis for to select flanking residues in larger peptides and proteins. Thus, the specification does provide significant guidance with regard to selection of further flanking residues even though the influence of sequences more distant from the cleavage site is less crucial to cleavage efficiency than the cleavage site proximal residues.

**(d) Genera of Peptide Substrates**

C8. As I explain above, a person of average skill in the field of the invention would understand from Table 6 and the surrounding text in the patent application that the inventors contemplated each one of the 2,940 permutations for $P_2P_1P_{1'}P_{2'}$ as defining an individual substrate sequence of their invention. It is equally clear that Table 6 as a whole defines a genus of 2,940 species of substrate having unique $P_2P_1P_{1'}P_{2'}$ sequences, and that the inventors contemplated this genus as part of their invention.

C9. It is my opinion that the patent application also conveys to the person of average skill in the field that other groupings of substrates (larger than one member and smaller than 2,940 members) also were contemplated as aspects of the invention. In fact, the inventors specifically identified in the application some of the groupings that they contemplated.

C10. For example, some genera are specifically defined by teachings in the application that the inventors had a preference for a smaller subset of amino acids, or a single amino acid, at one or more positions. In other words, if the inventors indicated that they preferred one of the ten position $P_2$ amino acids listed in Table 6, a person of average skill would understand from such teaching that the inventors contemplated a preferred genus of sequences defined by that one amino acid at position $P_2$ and any of the other amino acids set forth in Table 6 for positions $P_1$ $P_{1'}$ and $P_{2'}$ (6, 7, and 7, respectively) thereby defining a preferred genus of 1 x 6 x 7 x 7 = 294 sequences.

C11. There are many real examples in the application of these subgenera that the inventors specifically contemplated as part of their invention, where one or more residues are defined by fewer than all of the choices in Table 6. For example, on page 19, lines 8-11, the inventors identify some highly preferred amino acids that may be substituted in specific positions in $\beta$-secretase substrates. In particular, asparagine (N) is a preferred residue at $P_2$; residues Y, F and L are preferred at $P_1$; residues E, A and D are preferred at $P_{1'}$; and valine (V) is preferred at position $P_{2'}$. These excerpts of the application define multiple subgenera of $\beta$-secretase peptide substrates to a person of average skill in the field. For example, four subgenera are defined where one of the four positions of $P_2P_1P_{1'}P_{2'}$ have the more restricted definition, but the other three positions do not; six subgenera are defined where two of the positions have the restricted definition in the text and two do not; three subgenera are defined where three positions are restricted; and one subgenus is defined where all for positions of $P_2P_1P_{1'}P_{2'}$ have the restricted definition. This last subgenus has nine sequence permutations for $P_2P_1P_{1'}P_{2'}$ (1 x 3 x 3 x 1).

**(e) "Possession of the Invention": How to Make**

C12. By the year 2000, the ability to make peptide substrates was quite advanced. If a laboratory wanted a peptide of a specified sequence, it could provide the sequence to a technician who could make a reasonably pure quantity of the peptide using highly automated peptide synthesis machinery. Thus, the simple teaching of the necessary core peptide sequence in the patent application placed a person in the field "in possession" of the peptides per se.

### (f) Use of Peptides in Methods of the Invention

C13. The application describes practicing the methods of the invention with peptides or fusion proteins of the invention. A person of average skill would understand from such statements that the inventors contemplated using any or all of the peptides proteins that are defined as products of their invention in the methods of the invention. It would have been a matter of routine screening, using assays taught in the invention, to weed-out substrates in Table 6 with less than ideal activity, from the substrates that were readily cleaved by β-secretase enzyme.

## D. The Amended Claims Presented in each Application

D1. In general the pending claims in each of the applications concern isolated peptides that are cleaved by a human aspartyl protease and methods to assay for modulators of β-secretase activity by contacting a polypeptide with β-secretase activity with a substrate in the presence or absence of a putative modulator compound. The claimed peptide substrates are further defined as having (1) at least six amino acids, (2) a defined genus of residues are the $P_2$-$P_2$' positions flanking the β-secretase cleavage site, and (3) the ability to be cleaved by a specified human aspartyl protease. As discussed above the application details a number of assay methods to determine β-secretase activity and exemplifies such methods. Furthermore, the application describes specific sequences for β-secretase substrates that may be used in such methods. In view of the detailed disclosure regarding the peptide sequences and substrate sequences for use in claimed assay methods, it would have been recognized that the inventors were in possession of the presently claimed assay methods and the substrates for use therein.

### (i) The Claims in U.S. Patent Application No. 10/801,938

D2. The claims in U.S. Patent Application No. 10/801,938 concern assay methods employing any of a genus of modified APP molecules comprising a modified β-secretase processing site defined as follows: $P_2$ is N; $P_1$ is F; $P_1$' is A; or $P_2$' is A (see claim 84 from the '938 application in Exhibit B).

### (a) Definition of the Subgenus

D3. As explained above the specified amino acid residues at the $P_2$-$P_2$' position of the APP molecule are specifically set forth as some of the preferred residues for

each position in the description on page 5, lines 10-17 of the application and in Table 6 on page 30. The amino acids claimed at positions $P_2$, $P_1$ and $P_{1'}$ are coextensive with those listed in Table 6 on page 30. Furthermore, the specification discloses that optimized peptide substrates may comprise a A at the $P_{2'}$ position (*see, e.g.*, the Swedish mutation and page 24). A person of average skill would have understood that the teachings regarding a preferred value at $P_{2'}$ could and should be combined with teachings regarding the preferred Table 6 residues for position $P_2$, $P_1$ and $P_{1'}$ that claim 84 defines a subgenus of peptides that were specifically contemplated by the inventors. This subgenus of peptides may be tested using standard methods in the art to confirm that these peptides are preferred substrates. Moreover, the application specifically contemplates that some preferred substrates may be "mutant or derivative APP molecules in which the natural β-secretase cleavage site of wild-type APP has been modified . . ." (page 8, lines 4-6).

**(b) Studies concerning the subgenus**

D4. Experimental results in the applications indicated that larger substrates having additional amino terminal amino acid sequence, such as a modified APP molecule as recited in the claims of the '938 patent, are preferred because they exhibit enhanced cleavage rates (*see, e.g.*, results in Table 5 and on page 25, lines 18-20 of the specification). Furthermore, the application specifically demonstrates the functionality of peptide substrates that comprise N, L, K (see, wt APP), S, G, T (*see*, the ubiquitin amino acid sequence) D and A at the $P_2$ position; Y, L, M (*see, e.g.*, wt APP), Nle, and F at the $P_1$ position; E, A, D, M and S at the $P_{1'}$ position; and A at the $P_{2'}$ position (*see, e.g.*, Table 4 on page 24). Moreover polypeptides having predicted *β*-secretase cleavage sites that are analyzed by the inventors in Table 1 on pages 17-18, exemplify substrates having a E at position $P_2$ (one of the cleavage sites in oxidized insulin B-chain, SEQ ID NO: 37); Q at position $P_{1'}$ (the oxidized insulin A-chain, SEQ ID NO: 28); and G at position $P_{1'}$ (one of the cleavage sites in oxidized insulin B-chain, SEQ ID NO: 37 and two cleavage sites on the Notch polypeptides, SEQ ID NOS: 39 and 40).

D5. Studies published in International (PCT) Patent Publication No. WO 02/094985 demonstrate that a substrate have the claimed genus of $P_2P_1$-$P_{1'}P_{2'}$ as NF-AA is cleaved 17 time more efficiently than a peptide having the Swedish mutant App sequence

(SEQ ID NO: 259 in Table 3 on Page 42). This post-filing publication provides evidence of the functionality of the claimed substrates.

**(c) Conclusion**

D6. A person of average skill would have appreciated from teachings of the specification that the inventors were in possession of the claimed assay methods and the recited genus of modified APP substrates in the assay methods. It would have been expected that the vast majority of the substrates in the genus would be cleaved by $\beta$-secretase. Furthermore, in view of the detailed description regarding methods for screening $\beta$-secretase substrates, a skilled worker would recognize that only routine screening would be needed to determine whether any particular substrate defined in the claims would exhibit cleavage activity optimal for use in assays for $\beta$-secretase modulators.

**(ii) The Claims in U.S. Patent Application No. 10/801,493**

D7. The claims in U.S. Patent Application No. 10/801,493 concern assay methods employing a genus substrates at least six amino acids in length comprising a modified $\beta$-secretase cleavage site defined by the sequence $P_2P_1$-$P_{1'}P_{2'}$ wherein $P_2$ is N,; $P_1$ is F; $P_{1'}$ is E; and $P_{2'}$ is A. (See, for example, claim 84 of the '493 application in Exhibit C.)

**(a) Definition of the Subgenus**

D8. As detailed above, the inventors explicitly contemplated this subgenus of substrates: the residues at positions $P_2P_1$-$P_{1'}P_{2'}$ as defined in the claim were disclosed verbatim as the preferred amino acids for those positions in Table 6 on page 30, and at page 5, lines 10-17 of the specification.

**(b) Studies Concerning the Subgenus**

D9. The genus defined in Table 6 and repeated in claim 84 was deduced from the studies of peptide substrates that are detailed in the application. The application specifically demonstrates the functionality of peptides that comprise N, (see, wt APP) at the $P_2$ position; F at the $P_1$ position (a predicted $\beta$-secretase substrate also comprises a H at the $P_1$ position *see, e.g.*, SEQ ID NO: 33 at page 18 of the specification); E at the $P_{1'}$ position; and A at the $P_{2'}$ position (*see, e.g.*, Table 2 on page 20, Table 3 on page 21 and Table 4 on page 24).

D10. Studies published in International (PCT) Patent Publication No. WO 02/094985 demonstrate that a substrate have the claimed genus of $P_2P_1$-$P_1'P_2'$ as NF-EA is cleaved 17 time more efficiently than a peptide having the Swedish mutant App sequence (SEQ ID NO: 260 in Table 3 on Page 42). This post-filing publication provides evidence of the functionality of the claimed substrates.

**(c) Conclusions**

D11. A person of average skill would have appreciated from the teaching in the specification that the inventors were in possession of the recited genus of substrates for use in the assay methods claimed in the case. It would have been expected that the vast majority of the substrates in the genus would be cleaved by β-secretase. Furthermore, in view of the detailed description regarding methods for screening β-secretase substrates, a skilled worker would recognize that only routine screening would be needed to determine whether any particular substrate defined in the claims would exhibit cleavage activity optimal for use in assays for β-secretase modulators.

**(iii) The Claims in U.S. Patent Application No. 11/713,091**

D12. The claims in U.S. Patent Application No. 11/713,091 concern a genus of isolated peptides that are at least six amino acids in length comprising a modified β-secretase cleavage site defined by the sequence $P_2P_1$-$P_1'P_2'$ wherein $P_2$ is N, L, K, S, G, T, D, A, Q or E; $P_1$ is Y, L, M, Nle, F or H; $P_1'$ is E, A, D, M, Q, S or G; and $P_2'$ is A, N, T, L, F, S or V. (See, for example, claim 21 of the '091 application in Exhibit D.)

**(a) Definition of the Subgenus**

D13. As detailed above, the inventors explicitly contemplated this subgenus of substrates: the residues at positions $P_2P_1$-$P_1'P_2'$ as defined in the claim were disclosed verbatim as the preferred amino acids for those positions in Table 6 on page 30, and at page 5, lines 10-17 of the specification.

**(b) Studies Concerning the Subgenus**

D14. The studies presented in the application support the inventors' analysis that the genus defined in the preceding quotation comprises highly preferred β-secretase substrates. Specifically, the application demonstrates that 23 peptide sequences within the claimed genus [SEQ ID NO: 5 (NYEV), SEQ ID NO: 133 (NLEV), SEQ ID NO: 7 (NYAV),

SEQ ID NO: 46 (NYDV), SEQ ID NO: 47 (NLAV), SEQ ID NO: 48 (LY-AV), SEQ ID NO: 120 (TLEV), SEQ ID NO: 134 (NnleEV), SEQ ID NO: 135 (NnleEV), SEQ ID NO: 136 (NnleDV), SEQ ID NO: 137 (SLDV), SEQ ID NO: 138 (SLDV), SEQ ID NO: 143 (SYDA), SEQ ID NO: 144 (SYEA), SEQ ID NO: 141 (SYEV), SEQ ID NO; 145 (SYEV), SEQ ID NO: 147 (SYEV), SEQ ID NO: 148 (SYEV), SEQ ID NO: 149 (DYEV), SEQ ID NO: 150 (DTEV), SEQ ID NO: 151 (DYEV), SEQ ID NO: 152 (SYEV), SEQ ID NO: 153 (DYEV)] are cleaved by β-secretase (*see, e.g.,* Tables 2, 3, 4 and 5 on pages 20, 21, 24 and 25 of the specification respectively).

**(c) Conclusions**

D15. A person of average skill would have appreciated from the teaching in the specification that the inventors were in possession off the claimed assay methods when the applications were filed. It would have been expected that the vast majority of the substrates defined by the claimed subgenus would be cleaved by $\beta$-secretase. In any case, in view of the inventor's disclosure, it would have been a matter of routine screening to identify and weed-out inactive or suboptimal substrates.

**(iv) The Claims in U.S. Patent Application No. 11/753,331**

D16. The claims in U.S. Patent Application No. 11/753,331 concern a genus of isolated peptides that are at least six amino acids in length comprising a modified $\beta$-secretase cleavage site defined by the sequence $P_2P_1$-$P_{1'}P_{2'}$ wherein $P_2$ is N, S, or D; $P_1$ is Y, L or Nle; $P_{1'}$ is E, A, or D; and $P_{2'}$ is A or V. (See, for example, claim 21 of the '331 application in Exhibit E.)

**(a) Definition of the Subgenus**

D17. As detailed above, the inventors explicitly contemplated this subgenus of substrates: the residues at positions $P_2P_1$-$P_{1'}P_{2'}$ as defined in the claim were disclosed verbatim as the preferred amino acids for those positions in Table 6 on page 30, and at page 5, lines 10-17 of the specification.

**(b) Studies Concerning the Subgenus**

D18. The studies presented in the application support the inventors' analysis that the genus defined in the preceding quotation comprises highly preferred $\beta$-secretase substrates. Specifically, the application demonstrates that 21 peptide sequences within the

claimed genus [SEQ ID NO: 5 (NYEV), SEQ ID NO: 133 (NLEV), SEQ ID NO: 7 (NYAV), SEQ ID NO: 46 (NYDV), SEQ ID NO: 47 (NLAV), SEQ ID NO: 48 (LYAV), SEQ ID NO: 134 (NnleEV), SEQ ID NO: 135 (NnleEV), SEQ ID NO: 136 (NnleDV), SEQ ID NO: 137 (SLDV), SEQ ID NO: 138 (SLDV), SEQ ID NO: 143 (SYDA), SEQ ID NO: 144 (SYEA), SEQ ID NO: 141 (SYEV), SEQ ID NO: 145 (SYEV), SEQ ID NO: 147 (SYEV), SEQ ID NO: 148 (SYEV), SEQ ID NO: 149 (DYEV), SEQ ID NO: 151 (DYEV), SEQ ID NO: 152 (SYEV), SEQ ID NO: 153 (DYEV)] are cleaved by β-secretase (*see, e.g.,* Tables 2, 3, 4 and 5 on pages 20, 21, 24 and 25 of the specification respectively).

**(c) Conclusions**

D19. A person of average skill would have appreciated from the teaching in the specification that the inventors were in possession off the claimed assay methods when the applications were filed. It would have been expected that the vast majority of the substrates defined by the claimed subgenus would be cleaved by *β*-secretase. In any case, in view of the inventor's disclosure, it would have been a matter of routine screening to identify and weed-out inactive or suboptimal substrates.

## E. The State of the Art

**(i) References cited by the Examiner**

E1. A number of references have been cited by the Examiner that were published after the inventor's patent application was filed: Gruninger-Leitch *et al.* (*J. Biol. Chem.*, 277:4687-4693, 2002), Majer *et al.* (*Protein Science* 6: 1458-1466, 1997), Sauder *et al.* (*J. Mol. Biol.*, 300:241-248, 2000), Shi *et al.* (*J. Alzheimer's Disease* 7: 139-148, 2005), and Tomasselli *et al.* (*J. Neurochem.*, 84:1006-1017, 2003). I have reviewed each of these references and provide my comments below.

**(a) Gruninger-Leitch *et al.***

E2. The Examiner points to Gruninger-Leitch *et al.* as providing information about the state of the art at the time the application was filed, although it was published after the filing date. The authors of Gruninger-Leitch *et al.* used an experimental approach that is similar to that of the inventors, amino acid substitutions at cleavage site proximal residues to address the substrate specificity of *β*-secretase (BACE). Additionally,

cleavage of peptides from several random peptide libraries was examined to determine possible amino acid substitutions at various positions. In particular, the Examiner points out that Gruninger-Leitch *et al.* show that a single point mutation at the $P_{1'}$ or $P_4$ of the Swedish mutant cleavage site results in a drop in the rate of cleavage of the peptide substrate; nonetheless, the mutated substrates remained cleavable (see, Table 1 on page 4689). Essentially, what the studies presented in Gruninger-Leitch *et al.* show is that "BACE accepts a wide variety of peptidic substrates and, in contrast to other mammalian aspartic proteases, prefers acidic or polar residues at the P2 and P1' positions . . ." (page 4692, first column). This finding confirms what is disclosed by the inventors in the subject patent applications that are the subject of this analysis. The further studies by Gruninger-Leitch *et al.* indicate that the vast majority of the peptide substrates disclosed in Table 6 of the patent application will be cleaved by $\beta$-secretase.

**(b) Majer *et al.***

E3. The Examiner also cited Majer *et al.* 1997 indicating that Majer *et al.* shows evidence that residues further from the $\beta$-secretase cleavage site (*e.g.*, other than $P_2P_1$-$P_{1'}P_{2'}$) are of also important considerations that contribute to the cleavability of the substrate (Paragraph bridging Pages 8 and 9 of the Office Action). However, Majer *et al.* describes the development of inhibitors of the aspartyl protease cathepsin D based on site specificity. The amino acid substitutions described by Majer *et al.* concern the Pepstatin A peptide inhibitor compound. Therefore, the enzymatic activity measured in Majer *et al.* is inhibitory potency rather than cleavage by the protease. Because Majer *et al.* does not study protease substrates and also does not concern $\beta$-secretase, this reference does not provide any tangible information regarding peptide substrates disclosed and claimed in the patent applications currently under scrutiny. The conclusion in Gruninger-Leitch that "BACE accepts a wide variety of peptide substrates" is certainly more pertinent than Majer *et al.*'s analysis of peptide inhibitors of a different enzyme.

**(c) Sauder *et al.***

E4. Sauder *et al.*, a reference cited by the Examiner illustrates the interaction of a peptide substrate and an aspartyl protease at the enzymatic cleavages cite. Figure 4 of Sauder shows a six amino acid peptide centered at the cleavage site that spans the

BACE binding/cleavage pocket. The figure further indicates residues of BACE that interact with the $P_2$-$P_{2'}$ positions which illustrates why these residues are far more important to proteolytic cleavage as compared to residues at positions more distant to the cleavage site. Thus, Sauder *et al.* supports the opinion that I and persons of average skill in the field would share that the patent application has adequately described the structure of its substrates by focusing on the $P_2$ $P_1$ $P_{1'}$-$P_{2'}$ amino acids. (Of course, the application gives guidance for the structure at more distant resides as well.)

**(d) Tomasselli *et al.***

E5. Tomasselli *et al.* is a publication by some of the inventors of the patent applications and studies presented comprise data that is presented in the patent applications. As discussed above, the studies support the disclosure in the applications and show that the defined genera of peptide substrates are cleaved by *β*-secretase (*see, e.g.*, Table 1 on page 1010). Tomaselli *et al.*, also supports the conclusion that the addition of additional residues N- or C-terminal to the core sequence of substrate can enhance cleavage activity.

**(e) Shi *et al.***

E6. Shi *et al.* was published after the filing of the patent applications and provides further analyses regarding the cleavage of various *β*-secretase substrates. Similar to the inventors, the authors of Shi *et al.* focused on amino acid substitutions at the $P_2$-$P_{2'}$ positions (see Table 2 at page 142). Of the 24 peptides tested for cleavage activity, all but two were cleaved by β-secretase with equal or greater efficiency as compared to the wt-APP sequence. The studies in Shi *et al.* further confirm that the $P_2$-$P_{2'}$ positions of substrate peptides are the most important for cleavage efficiency and that broad range of substitutions can be made with out compromising the ability of a substrate to be cleaved. Specifically, Shi *et al.* concludes that "results of this present investigation further indicate that BACE1 can accept a wide variety of amino acid residues at the β-scissile-bond of its substrate both *in vitro* and in cells," (page 146, second column, second paragraph). The conclusions that were reached by Shi *et al.* further confirm that the inventor's disclosure regarding the β-secretase substrates was sufficient to support the claims that are pending in the applications under examination.

### (ii) Additional reference that characterize the state of the art

#### (a) Oliveira *et al.*

E7. Oliveira *et al.* (*Anal. Biochem.*, 203:39-46, 1992; Exhibit F) is a publication that concerns fluorometric methods for assessing protease activity. This reference is representative of knowledge that was available when the applications were filed. Oliveira *et al.* described the design of quenched fluorometric substrates for the protease rennin, which like β-secretase, is an aspartyl protease. Oliveira *et al.* demonstrate that a quenched fluorometric substrate having only six amino acids acts as a specific and efficient substrate for human rennin (see, Table 2 at page 43). Thus, at the time the application was filed, a person of average skill in the field would have recognized that peptides as small as six-amino acids in length were suitable protease substrates for aspartyl proteases. Furthermore, Oliveira *et al.* demonstrates that substrates of aspartyl proteases need not comprise additional residues outside of the immediate cleavage site to be efficiently cleaved. For this reason it would be expected that the most important amino acid positions in substrates would be those that flank the cleavage site (*e.g.*, $P_2$-$P_{2'}$). Thus, Olivera *et al.* supports my opinion, and that of persons of average skill in the field, that the patent application has adequately described the structure of the β-secretase substrates by focusing on the $P_2\,P_1\,P_{1'}P_{2'}$ amino acids.

#### (b) Andrau *et al.*

E8. Andrau *et al.* (*J. Biol. Chem*, 278:25859-25866, 2003; Exhibit G) was published after the patent applications were filed and describes the development of β-secretase assays using quenched fluorometric substrates. Andrau *et al.* further shows that the disclosure provided by the subject patent applications was sufficient with regard to the length β-secretase peptide substrates. For example, the specific substrates described by Andrau *et al.* (shown in figure 3 on page 25861) are six amino acids in length. Furthermore, the substrate sequences used correspond to wt-APP (JMV 2235) and Swedish mutant APP (JMV 2236) and both were shown to be effective to assess β-secretase activity. Thus, even the wt-APP sequence that is disclosed in the applicants to exhibit cleavage that is much less efficient than the Swedish mutant sequence is shown by Andrau *et al.* to be effective in assays for enzyme activity. Furthermore, Andrau *et al.* confirms that the cleavage site proximal amino acid positions are the most important for substrate cleavability. Specifically, the substrates of

Andau *et al.* only comprise the six positions flanking the cleavage site and thereby confirm that residues out side of this region are only peripherally involved in substrate cleavability. Thus, Andrau *et al.* provides further confirmation of the sufficient disclosure in the specification regarding peptide β-secretase substrates.

**(c) PCT Patent publication No. WO 02/094985**

E9. International (PCT) patent application published as WO 02/094985 was filed by Merck after the subject patent applications were filed and concerns β-secretase substrates. This publication confirms the teachings provided in the present application regarding β-secretase substrates that may be used in the claimed assay methods. In particular, the most active substrates identified in WO 02/094985 are listed on Table 3 at page 42 and these peptides have amino acid sequences that are coextensive with the substrate sequences indicated in Table 6 on page 30 of the subject specification. The most active of the substrates analyzed in the Merck application is cleaved 60 times more efficiently than a peptide corresponding the Swedish mutant APP sequence (SEQ ID NO: 262 in Table 3 on Page 42) and has as $P_2$-$P_{2'}$ sequence (NFEV) that is specifically described as a preferred substrate subgenus in the patent application (*see e.g.*, paragraph D14 above). Furthermore, the disclosure of the Merck application supports to opinion that peptides having a wide variety of sequences may be used as β-secretase substrates. For example, see Tables 2 on pages 36-39 (and descriptive text on page 11, lines 21-23) that lists 256 individual peptides that may be used as β-secretase substrates. Thus, WO 02/094985 also provides confirmation of the sufficient disclosure of β-secretase substrates in the specification.

## F. Conclusion

F1. In view of the foregoing comments it is my opinion that the claims set forth in the indicated U.S. patent applications (U.S. patent application nos. 10/801,938, 10/801,493, 11/713,091, 11/753,331) are sufficiently supported by the specification such that the skilled artisan would recognize that the inventors were in possession of the claimed invention at the time the applications were filed. I and ordinarily skilled artisans disagree with Examiner's assessment of what is fairly disclosed in the specification, what is demonstrated by the related literature and what would reasonably be concluded regarding untested peptides, based on existing evidence that BACE is tolerant of a wide array of peptide substrates.

F2. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of any U.S. patent issued in this application.

Date: 3/15/2010 By: John P. Anderson

John P. Anderson, Ph.D.

**CURRICULUM VITAE** **EXHIBIT A**

**John P. Anderson**

Elan Pharmaceuticals, Inc.
1000 Gateway Blvd.
South San Francisco, CA 94080

Telephone: 650-877-7422
Fax: 650-553-7196

john.p.anderson@elan.com

**Education**

**BA:** Haverford College (1971-1975)
Major: Chemistry
**Ph.D.:** University of Wisconsin- Madison (1975-1982)
Major: Pharmacology

**Positions held:**

**Elan Pharmaceuticals, Inc. Department of Biology;** Senior Scientist 2005-present, Principal Scientist 2001-2005, Scientist 1992-2001; Research: Isolation and characterization of beta-secretase (BACE); characterization of pathology-associated modifications of α-synuclein

**Mount Sinai Medical Center, Department of Psychiatry and Fishberg Center for Neurobiology;** Assistant Professor 1990-1992, Research Associate 1987-1990; Research: Expression and modifications of the Alzheimer amyloid precursor protein (APP)

**Columbia University, Department of Anatomy and Cell Biology;** Associate Research Scientist 1985-1987; Research: Estrogen and insulin effects on neural development

**Yale University School of Medicine, Department of Pathology;** Postdoctoral Fellow (NRSA Fellowship)1982-1985, Principal Investigator: Jon s. Morrow, M.D., Ph.D.; Research: Interaction of erythrocyte spectrin with calmodulin, using photoactivatable crosslinking reagents, and with ATP, using fluorescence and HPLC binding assays

**University of Wisconsin-Madison, Department of Pharmacology;** Graduate Research Assistant 1975-1982, Advisor: Arnold E. Ruoho, Ph.D.; Research: Spectrin interaction with the erythrocyte membrane, using synthesized photoactivatable crosslinking reagents

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Anderson, John P.; Basi, Guriqbal; Doan, Minh Tam; Frigon, Normand; John, Varghese; Power, Michael; Sinha, Sukanto; Tatsuno, Gwen; Tung, Jay; Wang, Shuwen; McConlogue, Lisa. **Human (small beta, Greek)-secretase enzyme, inhibitors and their compositions and uses.** *Publication Number- 2000047618/WO-A3; Publication Date- 2000-08-17*

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**secretase, and assays for detecting beta-secretase inhibition.** *Publication Number- 00871720/EP-A2; Publication Date- 1998-10-21*

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Chrysler, Susanna M. S.; Sinha, Sukanto; Keim, Pamela S.; Anderson, John P.; Jacobson-Croak, Kirsten L.; Tan, Hua; McConlogue, Lisa C. **'beta'-secretase, antibodies to 'beta'-secretase, and assays for detecting 'beta'-secretase inhibition.** *Publication Number- 1996040885/WO-A3; Publication Date- 1996-12-19*

**EXHIBIT B**

Pending Claims
10/801,938

84. A method for assaying for modulators of β-secretase activity, comprising:

(a) contacting a polypeptide with β-secretase APP processing activity with a substrate, both in the presence and in the absence of a putative modulator compound;

wherein said substrate comprises an APP molecule having a modified β-secretase processing site defined by formula $P_2P_1$-$P_{1'}P_{2'}$, wherein:

$P_2$-is N;

$P_1$ is F;

$P_{1'}$is A;

$P_{2'}$ is A;

wherein the substrate is cleaved between $P_1$ and $P_{1'}$ by a human aspartyl protease encoded by the nucleic acid sequence of SEQ ID NO: 1 or SEQ ID NO: 3 (Hu-Asp2); and

wherein said peptide does not comprise the corresponding $P_2P_1$-$P_{1'}P_{2'}$ portion of amino acid sequence depicted in SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, or SEQ ID NO: 39;

(b) measuring cleavage of the substrate peptide in the presence and in the absence of the putative modulator compound; and

(c) identifying modulators of β-secretase activity from a difference in substrate cleavage in the presence versus in the absence of the putative modulator compound, wherein a modulator that is a β-secretase antagonist reduces such cleavage and a modulator that is a β-secretase agonist increases such cleavage.

85. (previously presented) The method of claim 84,

wherein the modified β-secretase processing site is defined by formula $P_2P_1$-$P_{1'}P_{2'}P_{3'}$, and

wherein $P_{3'}$ comprises an amino acid selected from the group consisting of E, G, F, H, cysteic acid and S.

86. (canceled)

87. The method of claim 85, wherein the modified β-secretase processing site is defined by the formula $P_2P_1$-$P_{1'}P_{2'}P_{3'}$, wherein $P_{3'}$ is E.

88. The method of-claim 85, wherein the modified β-secretase processing site is defined by the formula $P_3P_2P_1$-$P_{1'}P_{2'}P_{3'}$, wherein $P_3$ is an amino acid selected from the group consisting of A, V, I, S, H, Y, T and F.

89. The method of claim 88, wherein $P_3$ comprises an amino acid selected from the group consisting of I or V.

90. The method of claim 88, wherein the modified β-secretase processing site is defined by the formula $P_4P_3P_2P_1$-$P_{1'}P_{2'}P_{3'}$ wherein $P_4$ is an amino acid selected from the group consisting of E, G, I, D, T, cysteic acid and S.

91. The method of claim 90, wherein the modified β-secretase processing site is defined by the formula $P_4P_3P_2P_1$-$P_{1'}P_{2'}P_{3'}P_{4'}$, wherein $P_{4'}$ is an amino acid selected from the group consisting of F, W, G, A, H, P, G, N, S, and E.

92 – 95. (canceled)

96. The method of any one of claims 84, 85 or 87-91 wherein the APP molecule further comprises a first label.

97. The method of claim 96 wherein the APP molecule further comprises a second label.

98. The method of any one of claims 84, 85 or 87-91 wherein the APP molecule further comprises a detectable label and a quenching moiety, wherein cleavage of the APP molecule between $P_1$ and $P_{1'}$ separates the quenching moiety from the label to permit detection of the label.

99. The method of claim 85, wherein said cysteic acid comprises a covalently attached label.

100. The method of any one of claims 84, 85 or 87-91, wherein the rate of cleavage of said APP molecule by said human aspartyl protease is greater than the rate of cleavage of a polypeptide comprising the human APP β-secretase cleavage sequence: SEVKMDAEFR (SEQ ID NO: 20).

101. The method of any one of claims 84, 85 or 87-91, wherein the rate of cleavage of said APP molecule by said human aspartyl protease is greater than the rate of cleavage of a polypeptide comprising the human APP Swedish KM→NL mutation, β-secretase cleavage sequence SEVNLDAEFR (SEQ ID NO: 19).

102. (currently amended) The method of any one of claims 84, 85 or 87-91, wherein the polypepetide with β-secretase APP processing activity comprises an amino acid sequence selected from the group consisting of

(a) the amino acid sequence of SEQ ID NO: 2,

(b) a fragment of the amino acid sequence of SEQ ID NO: 2 that retains β-secretase APP processing activity, wherein said fragment includes the aspartyl protease active site tripeptides DTG and DSG,

(c) an amino acid sequence that is at least 95% identical to (a) or (b), wherein the polypeptide includes the aspartyl protease active site tripeptides DTG and DSG and exhibits β-secretase APP processing activity;

(d) the amino acid sequence SEQ ID NO: 4,

(e) a fragment of the amino acid sequence of SEQ ID NO: 4 that retains β-secretase APP processing activity, wherein said fragment includes the aspartyl protease active site tripeptides DTG and DSG, and

(f) an amino acid sequence that is at least 95% identical to (d) or (e), wherein said fragment includes the aspartyl protease active site tripeptides DTG and DSG and exhibits β-secretase APP processing activity.

103. The method of any one of claims 84, 85 or 87-91, wherein the polypeptide with β-secretase APP processing activity comprises an amino acid sequence selected from the group consisting of

(a) the amino acid sequence of SEQ ID NO: 2; and

(b) a fragment of the amino acid sequence of SEQ ID NO: 2 that retains β-secretase APP processing activity, wherein said fragment includes the aspartyl protease active site tripeptides DTG and DSG.

104. A method according to claim 103, wherein the polypeptide with β-secretase APP processing activity comprises a polypeptide purified and isolated from a cell transformed or transfected with a polynucleotide comprising a nucleotide sequence that encodes the polypeptide.

105. A method according to claim 95,

wherein the APP molecule is expressed in a cell transformed or transfected with a polynucleotide comprising a nucleotide sequence that encodes the APP molecule,

wherein the cell expresses the polypeptide with β-secretase APP processing activity;

wherein the contacting comprises growing the cell in the presence and absence of the test agent, and

wherein the measuring step comprises measuring APP processing activity of the cell.

106. A method according to claim 105, wherein the contacting comprises administering the test agent to a transgenic non-human mammal that comprises the cell.

107. A method according to claim 84, wherein the polypeptide is encoded by a polynucleotide comprising the nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence of SEQ ID NO: 1 or SEQ ID NO; 3,

(b) a nucleotide sequence that hybridizes under the following stringent hybridization conditions to the complement of SEQ ID NO: 1 or 3:

(1) hybridization at 42°C in a hybridization buffer comprising 6x SSC and 0.1% SDS, and

(2) washing at 65°C in a wash solution comprising 1x SSC and 0.1% SDS;

wherein said nucleotide sequence encodes a polypeptide that exhibits β-secretase APP processing activity.

108. The method of claim 84, wherein the modified β-secretase processing site is defined by the formula $P_4P_3P_2P_1$-$P_{1'}P_{2'}P_{3'}P_{4'}$ as provided by SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 133, SEQ ID NO: 135, SEQ ID NO: 136, SEQ ID NO: 137, SEQ ID NO: 141, SEQ ID

NO: 143, SEQ ID NO: 144, SEQ ID NO: 145, SEQ ID NO: 147, SEQ ID NO: 149, SEQ ID NO: 150, SEQ ID NO: 151, SEQ ID NO: 152 or SEQ ID NO: 153.

109. (canceled)

110. The method of claim 88, wherein the peptide comprises a sequence of amino acids defined by the formula $P_3P_2P_1$-$P_{1'}P_{2'}P_{3'}$, wherein $P_3$ is V, $P_2$ is N, $P_1$ is F, $P_{1'}$ is A, $P_{2'}$ is A and $P_{3'}$ is E.

**EXHIBIT C**

Pending Claims
10/801,493

84. A method for assaying for modulators of β-secretase activity, comprising:

(a) contacting a polypeptide with β-secretase APP processing activity with a substrate, both in the presence and in the absence of a putative modulator compound;

wherein said substrate comprises a peptide having an amino acid sequence of at least 6 amino acids, said amino acid sequence including four amino acids defined by formula $P_2P_1$-$P_{1'}P_{2'}$, wherein:

$P_2$ is N;

$P_1$ is F ;

$P_{1'}$ is E;

$P_{2'}$ is A~~S~~;

wherein the substrate is cleaved between $P_1$ and $P_{1'}$ by a human aspartyl protease encoded by the nucleic acid sequence of SEQ ID NO: 1 or SEQ ID NO: 3 (Hu-Asp2); and

wherein said peptide does not comprise the corresponding $P_2P_1$-$P_{1'}P_{2'}$ portion of amino acid sequence depicted in SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, or SEQ ID NO: 39;

(b) measuring cleavage of the substrate peptide in the presence and in the absence of the putative modulator compound; and

(c) identifying modulators of β-secretase activity from a difference in substrate cleavage in the presence versus in the absence of the putative modulator

compound, wherein a modulator that is a β-secretase antagonist reduces such cleavage and a modulator that is a β- secretase agonist increases such cleavage.

85. The method of claim 84,

wherein said substrate comprises a peptide having an amino acid sequence of at least 6 amino acids, said amino acid sequence including five amino acids defined by formula $P_2P_1$-$P_1'P_2'P_3'$, and

wherein $P_3'$ comprises an amino acid selected from the group consisting of E, G, F, H, cysteic acid and S.

86-87. (canceled)

88. The method of claim 85, wherein the peptide comprises a sequence of amino acids defined by the formula $P_3P_2P_1$-$P_1'P_2'P_3'$, wherein $P_3$ is an amino acid selected from the group consisting of A, V, I, S, H, Y, T and F.

89. The method of claim 88, wherein $P_3$ comprises an amino acid selected from the group consisting of I or V.

90. The method of claim 88, wherein the peptide comprises a sequence of amino acids defined by the formula $P_4P_3P_2P_1$-$P_1'P_2'P_3'P_4'$ wherein $P_4$ is an amino acid selected from the group consisting of E, G, I, D, T, cysteic acid and S.

91. The method of claim 90, wherein the peptide comprises a sequence of amino acids defined by the formula $P_4P_3P_2P_1$-$P_1'P_2'P_3'P_4'$ wherein $P_4'$ is an amino acid selected from the group consisting of F, W, G, A, H, P, G, N, S, and E.

92-93. (canceled)

94. The method of claim 84, wherein said substrate comprises an amyloid precursor protein (APP) amino acid sequence with a modified β-secretase processing site defined by said formula $P_2P_1$-$P_{1'}P_{2'}$.

95. The method of any one of claims 84, 85 or 88-91, wherein said peptide comprises an amino acid sequence having up to 50 amino acids.

96. The method of any one of claims 84, 85, 88-91 or 94 wherein the peptide further comprises a first label.

97. The method of claim 96 wherein the peptide further comprises a second label.

98. The method of any one of claims 84, 85, 88-91 or 94 wherein the peptide further comprises a detectable label and a quenching moiety, wherein cleavage of the peptide between $P_1$ and $P_{1'}$ separates the quenching moiety from the label to permit detection of the label.

99. The method of claim 85, wherein said cysteic acid comprises a covalently attached label.

100. The method of any one of claims 84, 85, 88-91 or 94, wherein the rate of cleavage of said peptide by said human aspartyl protease is greater than the rate of cleavage of a polypeptide comprising the human APP β-secretase cleavage sequence: SEVKMDAEFR (SEQ ID NO: 20).

101. The method of any one of claims 84, 85, 88-91 or 94, wherein the rate of cleavage of said peptide by said human aspartyl protease is greater than the rate of cleavage of a polypeptide comprising the human APP Swedish KM→NL mutation, β-secretase cleavage sequence SEVNLDAEFR (SEQ ID NO: 19).

102. The method of any one of claims 84, 85, 88-91 or 94, wherein the polypepetide with β-secretase APP processing activity comprises an amino acid sequence selected from the group consisting of

(a) the amino acid sequence of SEQ ID NO: 2,

(b) a fragment of the amino acid sequence of SEQ ID NO: 2 that retains β-secretase APP processing activity, wherein said fragment includes the aspartyl protease active site tripeptides DTG and DSG,

(c) an amino acid sequence that is at least 95% identical to (a) or (b), wherein the polypeptide includes the aspartyl protease active site tripeptides DTG and DSG and exhibits β-secretase APP processing activity;

(d) the amino acid sequence SEQ ID NO: 4,

(e) a fragment of the amino acid sequence of SEQ ID NO: 4 that retains β-secretase APP processing activity, wherein said fragment includes the aspartyl protease active site tripeptides DTG and DSG, and

(f) an amino acid sequence that is at least 95% identical to (d) or (e), wherein said fragment includes the aspartyl protease active site tripeptides DTG and DSG and exhibits β-secretase APP processing activity.

103. The method of any one of claims 84, 85, 88-91 or 94, wherein the polypeptide with β-secretase APP processing activity comprises an amino acid sequence selected from the group consisting of

(a) the amino acid sequence of SEQ ID NO: 2; and

(b) a fragment of the amino acid sequence of SEQ ID NO: 2 that retains β-secretase APP processing activity, wherein said fragment includes the aspartyl protease active site tripeptides DTG and DSG.

104. A method according to claim 103, wherein the polypeptide with β-secretase APP processing activity comprises a polypeptide purified and isolated from a

cell transformed or transfected with a polynucleotide comprising a nucleotide sequence that encodes the polypeptide.

105. A method according to claim 95,

wherein the substrate is expressed in a cell transformed or transfected with a polynucleotide comprising a nucleotide sequence that encodes the substrate,

wherein the cell expresses the polypeptide with β-secretase APP processing activity;

wherein the contacting comprises growing the cell in the presence and absence of the test agent, and

wherein the measuring step comprises measuring APP processing activity of the cell.

106. A method according to claim 105, wherein the contacting comprises administering the test agent to a transgenic non-human mammal that comprises the cell.

107. A method according to claim 84, wherein the polypeptide is encoded by a polynucleotide comprising the nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence of SEQ ID NO: 1 or SEQ ID NO; 3,

(b) a nucleotide sequence that hybridizes under the following stringent hybridization conditions to the complement of SEQ ID NO: 1 or 3:

(1) hybridization at 42°C in a hybridization buffer comprising 6x SSC and 0.1% SDS, and

(2) washing at 65°C in a wash solution comprising 1x SSC and 0.1% SDS;

wherein said nucleotide sequence encodes a polypeptide that exhibits β-secretase APP processing activity.

108. A method according to claim 84, wherein the substrate comprises a peptide having an amino acid sequence selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 46, SEQ ID NO: 47, SEQ ID NO: 48, SEQ ID NO: 115, SEQ ID NO: 116, SEQ ID NO: 117, SEQ ID NO: 118, SEQ ID NO: 119, SEQ ID NO: 133, SEQ ID NO: 135, SEQ ID NO: 136, SEQ ID NO: 137, SEQ ID NO: 141, SEQ ID NO: 143, SEQ ID NO: 144, SEQ ID NO: 145, SEQ ID NO: 147, SEQ ID NO: 149, SEQ ID NO: 150, SEQ ID NO: 151, SEQ ID NO: 152 and SEQ ID NO: 153.

109. (canceled)

110. The method of claim 88, wherein the peptide comprises a sequence of amino acids defined by the formula $P_3P_2P_1$-$P_{1'}P_{2'}P_{3'}$, wherein $P_3$ is V, $P_2$ is N, $P_1$ is F, $P_{1'}$ is E, $P_{2'}$ is A and $P_{3'}$ is E.

**EXHIBIT D**

Pending Claims
11/713,091

14. (currently amended) The isolated peptide of claim [[1]] 21 comprising a first label.

15. (original) The isolated peptide of claim 14 further comprising a second label.

16. (currently amended) An isolated peptide according to claim [[1]] 21, further comprising a detectable label and a quenching moiety, wherein cleavage of the peptide between $P_1$ and $P_1'$ separate the quenching moiety from the label to permit detection of the label.

20. (currently amended) The isolated peptide of claim [[1]] 21, wherein said peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:5; SEQ ID NO:6; SEQ ID NO:7; SEQ ID NO:8; SEQ ID NO:9; SEQ ID NO:10; SEQ ID NO:11; SEQ ID NO:12; SEQ ID NO:13; SEQ ID NO:14; SEQ ID NO:15; SEQ ID NO:16; SEQ ID NO:17, SEQ ID NO:18; SEQ ID NO:120; SEQ ID NO:133; SEQ ID NO:134; SEQ ID NO:135; SEQ ID NO:136; SEQ ID NO:137; SEQ ID NO:138; SEQ ID NO:141; SEQ ID NO:143; SEQ ID NO:144; SEQ ID NO:145; SEQ ID NO:147; SEQ ID NO:148; SEQ ID NO:149; SEQ ID NO:150; SEQ ID NO:151; SEQ ID NO:152; SEQ ID NO:153; SEQ ID NO:154; SEQ ID NO:155; SEQ ID NO:156; SEQ ID NO:157; SEQ ID NO:158; SEQ ID NO:159; SEQ ID NO:160; SEQ ID NO:161; SEQ ID NO:162; SEQ ID NO:163; SEQ ID NO:164; SEQ ID NO:165; SEQ ID NO:166; SEQ ID NO:167; SEQ ID NO:168; SEQ ID NO:169; SEQ ID NO:190; SEQ ID NO:191; SEQ ID NO:192 and SEQ ID NO:193.

21. (currently amended) An isolated peptide comprising a sequence of at least ~~four~~ six amino acids defined by formula $P_2P_1$--$P_1'P_2'$, wherein:

$P_2$ comprises an amino acid selected from the group consisting of N, S, L, K, G, T, A, Q, E and D;

$P_1$ comprises an amino acid selected from the group consisting of Y, L, M, F, H and Nle;

$P_1'$ comprises an amino acid selected from the group consisting of E, A, M, Q, S, G and D;

$P_2'$ comprises an amino acid selected from the group consisting of A, N, T, L, F and V; and

wherein a human Aspartyl protease encoded by the nucleic acid sequence of SEQ ID NO: 1 or SEQ ID NO: 3 (Hu-Asp2) cleaves said peptide between $P_1$ and $P_1'$; and

~~with the proviso that if $P_1'P_2'$ comprise the sequence DA, $P_2P_1$ do not comprise the sequences NL or NNle~~.

wherein said peptide is cleaved between $P_1$ and $P_1'$ by a human aspartyl protease encoded by the nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:3 and said peptide does not comprise the corresponding $P_2P_1$--$P_1'P_2'$ portion of amino acid sequences depicted in SEQ ID NO:19; SEQ ID NO:20; SEQ ID NO:21; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:28; SEQ ID NO:31; SEQ ID NO:32; SEQ ID NO:33; SEQ ID NO:34; SEQ ID NO:35; SEQ ID NO:36; SEQ ID NO:37; SEQ ID NO:38; SEQ ID NO:39; or SEQ ID NO:40.

27. (original) A polypeptide comprising a peptide sequence according to claim 21, and further comprising a transmembrane domain to localize the polypeptide to a cellular membrane when the polypeptide is expressed in a eukaryotic cell.

28. (original) A polypeptide comprising a peptide according to claim 1 and further comprising a transmembrane domain amino acid sequence.

32. (original) The polypeptide according to claim 28, wherein said transmembrane domain anchors said polypeptide to an intracellular membrane selected from the group consisting of the Golgi or the endoplasmic reticulum.

**EXHIBIT E**

Pending Claims
11/753,331

21. An isolated peptide comprising an amino acid sequence of at least six amino acids defined by formula $P_2P_1$--$P_1'P_2'$, wherein:

$P_2$ comprises an amino acid selected from the group consisting of N, S, and D;

$P_1$ comprises an amino acid selected from the group consisting of Y, L, and Nle;

$P_1'$ comprises an amino acid selected from the group consisting of E, A, and D;

$P_2'$ comprises an amino acid selected from the group consisting of A and V; and

wherein a human Aspartyl protease encoded by the nucleic acid sequence of SEQ ID NO: 1 or SEQ ID NO: 3 (Hu-Asp2) cleaves said peptide between $P_1$ and $P_1'$;

wherein said peptide is cleaved between $P_1$ and $P_1'$ by a human aspartyl protease encoded by the nucleic acid sequence of SEQ ID NO: 1 or SEQ ID NO: 3 and said peptide does not comprise the corresponding $P_2P_1$-$P_1'P_2'$ portion of amino acid sequence depicted in SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 31, SEQ ID NOl 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39 or SEQ ID NO: 40.

23. An isolated peptide according to claim 21, wherein the Hu-Asp2 cleaves the peptide at a rate greater than the Hu-Asp2 cleaves a corresponding peptide having the $P_2P_1$--$P_1'P_2'$ amino acid sequence KMDA.

27. A polypeptide comprising a peptide sequence according to claim 21, and further comprising a transmembrane domain to localize the polypeptide to a cellular membrane when the polypeptide is expressed in a eukaryotic cell.

73. An isolated peptide comprising a sequence of at least 10 amino acids having the sequence SEISY-EVEFR (SEQ ID NO:152).

Exhibit F

ANALYTICAL BIOCHEMISTRY 203, 39–46 (1992)

# Intramolecularly Quenched Fluorogenic Peptide Substrates for Human Renin[1]

Maria C. F. Oliveira,* Izaura Y. Hirata,* Jair R. Chagas,* Paulo Boschcov,* Roseli A. S. Gomes,† Amintas F. S. Figueiredo,† and Luiz Juliano*

*Department of Biophysics, Escola Paulista de Medicina, Rua 3 de Maio, 100, Sao Paulo, 04044 Brazil; and †Department of Biochemistry and Immunology, Universidade Federal de Minas Gerais, Brazil

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**Six intramolecularly quenched fluorogenic peptides related to the sequences Phe[8] to His[13], His[6] to His[13], and Tyr[4] to His[13] of the human angiotensinogen, containing o-aminobenzoyl (Abz) and ethylenediamine dinitrophenyl (EDDnp) groups at amino- and carboxyl-terminal amino acids residues, were synthesized by classical solution methods. The Leu–Val is the only bond of all obtained peptides that was hydrolyzed by human renin with different degrees of purity and was resistant to hydrolysis by pig renin and cathepsin D. The hydrolysis of Abz–His–Pro–Phe–His–Leu–Val–Ile–His–EDDnp by human renin was inhibited by a highly specific transition-state analog of angiotensinogen ($IC_{50}$ = 7.8 × $10^{-9}$ M), described by K. Iizuka *et al.* (1990, *J. Med. Chem.* 33, 2707–2714). Therefore, specific and sensitive substrates for the continuous assay of human renin in which as little as 70 μGU of human renin could be detected by Abz–Phe–His–Leu–Val–Ile–His–EDDnp were described. The optimal pHs of hydrolysis of the substrates were in the range 4 to 6.** 

Renin (EC 3.4.23.15) is a special aspartyl protease from both the physiological and enzymatic points of view, because it is involved in the initial step of the renal vasopressor system, releasing angiotensin I through a very specific cleavage of angiotensinogen at pH near neutrality. The N-terminal tetradecapeptide sequence of equine angiotensinogen (Asp[1]–Arg[2]–Val[3]–Tyr[4]–Ile[5]–His[6]–Pro[7]–Phe[8]–His[9]–Leu[10]–Leu[11]–Val[12]–Tyr[13]–Ser[14]) is substrate for several mammalian renins (1–3). However, human angiotensinogen is a specific substrate for primate renins (1) and its N-terminal tetradecapeptide sequence (Asp[1]–Arg[2]–Val[3]–Tyr[4]–Ile[5]–His[6]–Pro[7]–Phe[8]–His[9]–Leu[10]–Val[11]–Ile[12]–His[13]–Asn[14]) (4) differs from the equine sequence in the last four residues. The scissile bonds are Leu[10]–Val[11] in human and Leu[10]–Leu[11] in equine angiotensinogens.

The first procedures employed for evaluation of renin activity were the biological assays, based on the rapid *in vivo* conversion of angiotensin I to the very active vasopressor angiotensin II (5). Later, the radioimmunoassay of angiotensin I (6) became more widely used for the evaluation of active renin concentrations in biological samples. Nonradioactive assays based on the activity of renin upon the synthetic angiotensinogen (1–14) and quantitation of the released C-terminal tetrapeptide (Leu–Val–Tyr–Ser) by Lowry reagent (7), fluorescamine (8), or antraquinone derivative (9) were also developed.

Fluorimetric assays in which [2]Z–Pro–Phe–His–Leu–Leu–Val–Tyr–Ser–β-naphthylamide (10), Arg–Pro–

[1] This research was supported by the Brazilian research agencies FINEP, CNPq, FAPESP, and FAPEMIG.

[2] Abbreviations used: nomenclature for amino acids and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (*Biochemistry* **5**, 3485, 1966; **6**, 362, 1967; **11**, 1726, 1972); Abz, *o*-aminobenzoyl; Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DCU, dicyclohexylurea; DMF, dimethylformamide; AcOH, acetic acid; AcOEt, ethyl acetate; MeOH, methanol; DMSO, dimethyl sulfoxide; ACN, acetonitrile; DIPEA, diisopropylethylamine; BOP, benzotriazol-yloxytris(dimethylamino)phosphonium hexafluorophosphate; Tos, tosyl; EDDnp, ethylenediamine dinitrophenyl; TEA, triethylamine; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl; SDS, sodium dodecyl sulfate; AMC, 7-amino-4-methylcoumarine; Z-Abz-ONSu, *o*-(benziloxycarbonyl-amino)-benzoyl-*N*-hydroxysuccinimide ester; PMSF, phenylmethanesulfonyl fluoride; GU, Goldblatt Unit [as defined by Haas *et al.*, *Lancet*, **657**, 1968, using the renin standard from Medical Research Council (London, UK)]; μGU, GU × $10^{-6}$.

0003-2697/92 $5.00

Phe–His–Leu–Leu–Val–Tyr–AMC, or its succinilated derivative (11) is used as substrate were described. The C-terminal-released peptides, Leu–Val–Tyr–Ser–β-naphthylamide and Leu–Val–Tyr–AMC, were then sequentially digested by an aminopeptidase, yielding the fluorescent β-naphthylamine or coumarin derivative. Renin assays based on HPLC separation and quantitation of substrates and their products of hydrolysis were also employed (12, 13). However, all the procedures described so far for evaluation of renin activity suffer some limitation related to sensitivity or specificity or are time consuming.

Continuous fluorimetric assays for endopeptidases, using intramolecularly quenched fluorogenic substrates, were developed by Yaron *et al.* (14) and have been widely used in assays of collagenase and gelatinase (15), aminopeptidase (16), HIV protease (17–19), several neutral proteases (20), endooligopeptidases (21,22), pepsin (23,24), and kallikreins (25).

The fluorescence increase during the hydrolysis of an intramolecularly quenched fluorogenic substrate is very sensitive to the distance between the donor and the acceptor groups. We had previously obtained significant indications that the equine angiotensinogen (6–8) sequence (His–Pro–Phe–His) is involved in a β-turn structure in aqueous solution (26), which was later confirmed by X-ray analysis (27). These observations prompted us to synthesize and assay, as human renin substrates, peptides related to segment 4–13 of human angiotensinogen containing *ortho*-aminobenzoic (Abz) as the fluorescent group and ethylenediamine-(2,4)-dinitrophenyl (EDDnp) as the quencher group at the amino- and carboxyl-terminal positions, respectively. The specificity of the obtained substrates was evaluated by studying their hydrolysis by human renin preparations with different degrees of purity, by porcine renin, and also by an acidic protease isolated during human renin purification, which presents properties very similar to those of cathepsin D.

## MATERIALS AND METHODS

### Enzyme

Human renin was kindly supplied by Dr. E. Haas (Mount Sinai Hospital, Cleveland, OH) and used to determine the kinetic parameters. A renin preparation with a specific activity of 880 GU/mg protein was also obtained from human kidney cortex as follows: A crude extract obtained from defatted kidney powder, treated with 10 mM $NaH_2PO_4/Na_2HPO_4$ (pH 6.5) containing 0.8 mM PMSF, 1 mM EDTA, 0.4 mM tetrathionate, and 30% (v/v) 2-methoxyethanol, was applied successively to pepstatin-A–aminohexyl–Sepharose, reactive blue-agarose, and CM–Sepharose columns. In this last chromatography, an acidic protease with characteristics very similar to those of cathepsin D was separated from renin. Both enzymes were homogeneous on SDS electrophoresis and analytical gel filtration, with renin presenting a molecular weight of 39,000 and the acidic protease one of 36,000. This enzyme released angiotensin I from the equine angiotensinogen (1–14) and presented a specific activity with bovine hemoglobin, at pH 3.0 and 37°C, of 2.4 mg of tyrosine $h^{-1}$ $mg^{-1}$. Both purified enzymes were obtained in small amounts and used only to characterize the cleavage site of the synthetic fluorogenic substrates by HPLC. The scaling up of the preparations of these enzymes are in progress and details of the purification procedures and enzyme characterization will be presented elsewhere.

### Peptide Synthesis

The methods employed were essentially the same as those previously reported (25,28), and the descriptions given below include details that have not been published before. Thin-layer chromatography was carried out on 0.25-mm silica gel (Kieselgel, Merck) plates and $R_f(A)$, $R_f(B)$, $R_f(C)$, and $R_f(D)$ values refer, respectively, to the solvent systems *n*-BuOH–AcOH–$H_2O$ (4:1:1), *n*-BuOH–pyridine–AcOH–$H_2O$ (30:20:6:24), AcOEt–EtOH (4:1), and $CHCl_3$–MeOH (98:2). Amino acid compositions of acid hydrolysates (6 N HCl, 110°C, 72 h) were determined using a Beckman high-performance amino acid analyzer (Model 6300). The melting points were measured with a Reichert micro hot stage apparatus and are uncorrected. The final deprotected peptides were purified to at least 95% purity through a semipreparative HPLC as follows: Econosil column—$C_{18}$, 10 μm, 22.5 × 250 mm; solvents—(A) TFA/$H_2O$ (1:1000), (B) TFA/(ACN + $H_2O$) (1:900:100); flow—5 ml/min; gradient—30–50% B in 30 min. $R_t$ is the retention time in analytical HPLC as follows: Ultrasphere column—$C_{18}$, 5 μm, 4.6 × 150 mm; solvents—(A) TFA/$H_2O$ (1:1000), (B) TFA/(ACN + $H_2O$) (1:900:100); flow—1.0 ml/min; gradient—10–80% B in 15 min and additional 5 min at 80% B. Detection for all HPLC analyses was uv = 220 nm; fluorescence excitation, 320 nm; emission, 420 nm.

### *Synthesis of Abz–Phe–His–Leu–Val–Ile–His–EDDnp* (I)

*Z–Ile–His–EDDnp* (**a**). This compound was obtained by conversion of Z–Ile–His–$NHNH_2$ (29) to the corresponding azide, following the general method previously described (30), and coupling with EDDnp · HCl (31). Z–Ile–His–$NHNH_2$ (14 mmol), HCl (50 mmol from a 4 N solution in dioxane), and isoamyl nitrite (16 mmol) were dissolved in DMF at −20°C. The disappearance of hydrazide (15 min) was checked by TLC with system D, developed by 0.3 M $FeCl_3$–0.2 M $K_3Fe(CN)_6$ (1:1) in

AcOH. The solution was further cooled to −60°C, its pH was adjusted to 7.5 with $Et_3N$, and it was added to a solution of EDDnp (16 mmol) in DMF precooled to −10°C. This reaction mixture was stirred at 0°C for 5 h and at 4°C for additional 48 h. During the course of the reaction, the pH was carefully maintained between 7.0 and 7.5 by adding $Et_3N$. After the pH was brought to 5.0 with AcOH, the solvent was removed by evaporation and 5% $NaHCO_3$ was added to the residue, resulting in a precipitate, which was collected by filtration and washed with 5% $NaHCO_3$, water, 2% AcOH, and water. The resulting material was dissolved in MeOH and reprecipitated with water. Yield: 89%; mp 159–163°C; $R_f(A)$ 0.69, $R_f(B)$ 0.85, $R_f(C)$ 0.72, $R_f(D)$ 0.74.

*Z–Val–Ile–His–EDDnp* (**b**). This compound was obtained by the mixed anhydride procedure as follows. *N*-Methylmorpholine (13.3 mmol) and isobutyl chloroformate (12.2 mmol) were added to a precooled (−15°C) solution of Z–Val–OH (13.3 mmol) in 100 ml of DMF. After 5 min a solution of Ile–His–EDDnp · HBr (prepared from 11.2 mmol of compound **Ia** and 15 ml of 2.6 N HBr/AcOH) in DMF, precooled at −15°C, was added, and the pH adjusted to 7.5 with $Et_3N$. The reaction mixture was stirred at −10°C for 1 h and 4°C overnight. Then 2.5 M $KHCO_3$ (4 mmol) was added and stirred for 30 min, in order to destroy the excess of mixed anhydride. The solvent was removed by evaporation, and 5% $NaHCO_3$ was added to the residue to form a precipitate, which was collected by filtration and processed as described for compound **Ia**. Yield: 79%; mp 193–197°C; $R_f(A)$ 0.61, $R_f(B)$ 0.88, $R_f(C)$ 0.77, $R_f(D)$ 0.44.

*Z–Leu–Val–Ile–His–EDDnp* (**c**). This compound was obtained from Z–Leu–OH (9.6 mmol) and Val–Ile–His–EDDnp · HBr (prepared from 7.4 mmol of compound **Ib** and 15 ml of 2.6 N HBr/AcOH) by the mixed anhydride procedure as described for compound **Ib**. Yield: 91%; mp 212–217°C; $R_f(A)$ 0.60, $R_f(B)$ 0.73, $R_f(C)$ 0.78, $R_f(D)$ 0.50.

*Z–Phe–His–NHNH₂* (**d**). Z–Phe–His–OMe was obtained by coupling Z–Phe–OH (60 mmol) to His–OMe · 2HCl (60 mmol) by the mixed anhydride procedure as described for compound **Ib**. After concentration of the reaction mixture, the residue was dissolved in AcOEt–water and the organic phase was washed with 5% $NaHCO_3$, water–NaCl, dried over $Na_2SO_4$, and crystallized from MeOH–ether. Yield: 84%; mp 124–128°C; $R_f(A)$ 0.62, $R_f(B)$ 0.62, $R_f(D)$ 0.57. Hydrazine hydrate (4.8 ml) was added to a solution of Z–Phe–His–OMe (20 mmol) in MeOH (40 ml). After 24 h at room temperature the crystallized hydrazide was filtered, washed with cold MeOH–ether, and dried in vacuum over concentrated $H_2SO_4$. Yield: 70%; mp 178–180°C; $R_f(A)$ 0.45, $R_f(B)$ 0.61, $R_f(D)$ 0.10. Amino acid analysis: His, 0.98; Phe, 1.02 (average recovery 84%).

*Z–Phe–His–Leu–Val–Ile–His–EDDnp* (**e**). This compound was obtained by coupling Z–Phe–His–$N_3$ (prepared from 6.4 mmol of compound **Id**) to Leu–Val–Ile–His–EDDnp · HBr (prepared from 6.5 mmol of compound **Ic** and 15 ml of 2.6 N HBr/AcOH), using the same procedure described for compound **Ia**. The final product was reprecipitated from DMF–water. Yield: 68%; mp 243–246°C; $R_f(A)$ 0.36, $R_f(B)$ 0.70, $R_f(C)$ 0.64, $R_f(D)$ 0.31.

*Abz–Phe–His–Leu–Val–Ile–His–EDDnp* (**f**). This compound was obtained by coupling Phe–His–Leu–Val–Ile–His–EDDnp (prepared from 0.28 mmol of compound **Ie** and 4.5 ml of 2.6 N HBr/AcOH) with benzotriazol-1-yl-*o*-aminobenzoate (Abz-OBt) (32) in DMF (2 ml), with the pH adjusted to 7.5 with TEA. After 24 h at room temperature, under stirring, the solvent was evaporated and the compound precipitated by addition of 5% $NaHCO_3$, which was filtered, washed with 5% $NaHCO_3$, water, and AcOEt, and reprecipitated from DMF–AcOEt. Yield: 68%; mp 253–256°C; $R_f(A)$ 0.57, $R_f(B)$ 0.78, $R_f(C)$ 0.71. Thirty milligrams of this material was purified by semipreparative HPLC with recovery of 16 mg. $R_t$ = 16.2 min. Amino acid analysis: His, 2.05; Val, 0.96; Ile, 0.96; Leu, 1.02; Phe, 1.03 (average recovery 67.5%). $R_f(A)$ 0.56, $R_f(B)$ 0.76, $R_f(C)$ 0.70.

*Synthesis of Abz–His–Pro–Phe–His–Leu–Val–Ile–His–EDDnp* (**II**)

Z–His–Pro–Phe–His–Leu–Val–Ile–His–EDDnp, prepared from compound **Ie** and Z–His–Pro–OH (33) with BOP-reagent, was deprotected by 2.6 N HBr/AcOH and treated with Abz-OBt as described for compound **If**. Yield: 73%. Thirty milligrams of obtained peptide was purified by HPLC, with a recovery of 14 mg (40%). $R_t$ = 14.7 min. Amino acid analysis: Pro, 0.99; Val, 0.92; Ile, 1.05; Leu, 1.08; Phe, 1.02; His, 2.85 (average recovery 88%). $R_f(A)$ 0.13, $R_f(B)$ 0.56, $R_f(C)$ 0.63.

*Synthesis of Abz–His–Gly–Phe–His–Leu–Val–Ile–His–EDDnp* (**III**)

This compound was obtained similarly as described for compound **II**. Yield: 80%. Twenty-five milligrams of this material was purified by HPLC with a recovery of 13 mg. $R_t$ = 14.5 min. Amino acid analysis: Gly, 0.96; Val, 0.91; Ile, 0.92; Leu, 1.02; Phe, 0.95; His, 3.10 (average recovery 78%). $R_f(A)$ 0.11, $R_f(B)$ 0.49, $R_f(C)$ 0.61.

*Synthesis of Abz–Arg–Pro–Phe–His–Leu–Val–Ile–His–EDDnp* (**IV**)

Abz–Arg(Tos)–Pro–Phe–His–Leu–Val–Ile–His–EDDnp, obtained as described for compound **II** was treated with anhydrous HF and anisole at 0°C for 1 h. After evaporation of HF, the resulting oil was dissolved

in 20% AcOH, washed with ether, and lyophilized. Forty milligrams of this material was purified by HPLC, with a recovery of 26 mg. $R_t$ = 14.7 min. Amino acid analysis: Arg, 1.05; Pro, 0.97; Val, 0.99; Ile, 0.92; Leu, 1.06; Phe, 1.01 (average recovery 74%). $R_f(A)$ 0.32, $R_f(B)$ 0.55, $R_f(C)$ 0.60.

*Synthesis of Abz–Arg–Gly–Phe–His–Leu–Val–Ile–His–EDDnp* (**V**)

The Arg(Tos)-protected peptide was prepared and treated with anhydrous HF as described for compound **IV.** Fifty milligrams of this lyophilized material was purified by HPLC, with a recovery of 25 mg. $R_t$ = 14.6 min. Amino acid analysis: Gly, 1.01; Val, 0.94; Ile, 0.96; Leu, 0.96; Phe, 1.03; His, 2.08 (average recovery 76%). $R_f(A)$ 0.25, $R_f(B)$ 0.58, $R_f(C)$ 0.65.

*Synthesis of Abz–Tyr–Ile–His–Pro–Phe–His–Leu–Val–Ile–His–EDDnp* (**VI**)

*Z–Abz–Tyr–Ile–His–Pro–NHNH–Boc* (**a**). This compound was synthesized by reacting Tyr–Ile–His–Pro–NHNH–Boc [obtained from 1.9 mmol of Z–Tyr–Ile–His–Pro–NHNH–Boc (34) by treatment with $H_2$/Pdl with Z–Abz–ONSu (35) (1.9 mmol) in DMF (8 ml). After 24 h at room temperature and pH adjustment to 7.5 with TEA, the compound was precipitated by the addition of water, isolated by filtration, and washed with 5% $NaHCO_3$, 2% AcOH, and water. Yield: 93%; mp 149–151°C; $R_f(A)$ 0.63, $R_f(B)$ 0.94, $R_f(C)$ 0.83, $R_f(D)$ 0.53.

*Z–Abz–Tyr–Ile–His–Pro–Phe–His–Leu–Val–Ile–His–EDDnp* (**b**). This compound was obtained by converting Z–Abz–Tyr–Ile–His–Pro–$NHNH_2$ (obtained from 0.44 mmol of compound **VIa** by treatment with 4 ml of 3 N HCl/dioxane) to the corresponding azide and coupling to Phe–His–Leu–Val–Ile–His–EDDnp · HBr (0.22 mmol) as described for compound **Ia.** The product was isolated by precipitation from EtOH–water and washed in the filter with the same solvent and 2% AcOH. Yield: 93%; mp 189–193°C; $R_f(A)$ 0.35, $R_f(B)$ 0.69, $R_f(C)$ 0.78.

*Abz–Tyr–Ile–His–Pro–Phe–His–Leu–Val–Ile–His–EDDnp* (**c**). This compound was obtained by treatment of compound **VIb** (0.2 mmol) with 4.5 ml of 2.6 N HBr/AcOH for 1 h at room temperature. The peptide was precipitated from ether and dried over solid KOH. Twenty milligrams of this material was purified by HPLC, with recovery of 6.5 mg. Amino acid analysis: Pro, 1.10; Val, 1.20; Ile, 1.88; Leu, 0.88; Tyr, 0.80; His, 2.95 (average recovery 68%). $R_f(A)$ 0.25, $R_f(B)$ 0.71, $R_f(C)$ 0.72.

*Syntheses of Abz–Phe–His–Leu and Abz–X–Phe–His–Leu* (**VII**) *(X = His–Pro, His–Gly, Arg–Pro, Arg–Gly, Tyr–Ile–His–Pro)*

All these peptides were obtained by the same strategies employed in the syntheses described above starting from Z–Phe–His–Leu–OtBut, which was prepared by reaction of Z–Phe–His–$N_3$ (12.8 mmol) and Leu–OtBut (25 mmol) as described for compound **Ie.** The analytical data of the final products were as expected.

HPLC Analysis of the Synthetic Substrates and Their Enzymatic Hydrolysis Products

The substrate solutions in 100 mM phosphate buffer (pH 5.5), containing 10% DMSO, were incubated with enzyme preparations at 37°C. Samples (20 μl) of the enzymatic digests were removed for analysis until 100% of substrate hydrolysis was reached. Samples of the substrates and enzyme controls were also analyzed. The products of hydrolysis were identified by simultaneous injection of the enzymatic digests with the corresponding authentic synthetic products. The HPLC conditions were the same as those described above for analytical purposes.

This general procedure was applied to all obtained substrates using Haas and purified renin. Figure 1 shows the HPLC profiles of compounds **I** and **VI** incubated with Haas renin. The digestion products of compound **I** by the crude extract of kidney powder, described above, were also analyzed by HPLC. The isolated acid protease was assayed with all substrates in 100 mM formiate (pH 3.0) and 100 mM phosphate (pH 5.5) buffers and no hydrolysis was detected. Pig renin, at 0.1 GU/ml, was assayed under the same conditions employed with human renin and no hydrolysis was detected. Five micrograms per milliliter of porcine pepsin (Sigma, St. Louis, Lot 15B-1370) hydrolyzed completely all substrates at the Leu–Val bond, after 2 h of incubation in 100 mM sodium acetate buffer (pH 4.0), at 37°C.

Fluorimetric Enzyme Assay

The hydrolysis of each substrate by renin was monitored by measuring the fluorescence at $\lambda_{em}$ = 420 nm and $\lambda_{ex}$ = 320 nm in a Perkin–Elmer spectrofluorometer (Model LC-5). These are the optimal fluorimetric conditions obtained from the excitation and emission spectra of all substrates studied. The standard conditions for hydrolysis at 37°C were 100 mM sodium acetate buffer (pH 5.5) containing 0.1% gelatine and 10% DMSO. The 1-cm path length cuvette containing 950 μl of the mixture of buffer and substrate solution (previously dissolved in DMSO) was left in the thermostated cell compartment until temperature equilibrium of the solution was attained (5–10 min). During this time no

TABLE 1

Fluorescence Increase after Total Hydrolysis of Quenched Fluorogenic Substrates for Human Renin

| No. | Peptides | $F_f/F_i$ |
|---|---|---|
| I | Abz-Phe-His-Leu-Val-Ile-His-EDDnp | 23 |
| II | Abz-His-Pro-Phe-His-Leu-Val-Ile-His-EDDnp | 16 |
| III | Abz-His-Gly-Phe-His-Leu-Val-Ile-His-EDDnp | 29 |
| IV | Abz-Arg-Pro-Phe-His-Leu-Val-Ile-His-EDDnp | 27 |
| V | Abz-Arg-Gly-Phe-His-Leu-Val-Ile-His-EDDnp | 33 |
| VI | Abz-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-EDDnp | 7 |

*Note.* The fluorescence ($F_i$) of the peptide solutions in 100 mM sodium acetate buffer (pH 4.0) was measured in a spectrofluorimeter ($\lambda_{em}$ = 420 nm and $\lambda_{ex}$ = 320 nm). Pepsin (5 μg/ml) was added and the fluorescences were measured until a constant value was reached ($F_f$).

significant change in fluorescence was detected. The enzyme was then added and the increase in fluorescence with time was continuously recorded for 5–20 min, depending on the rate of substrate hydrolysis. The slope was converted into moles of substrate hydrolyzed per minute, using a calibration curve for each substrate obtained from the corresponding synthetic fluorescent fragment released during the hydrolysis. The enzyme concentrations used allowed initial rate determinations of less than 5% of the total substrate. Although the water solubility of all studied substrates was not high and demanded addition of DMSO, the substrate concentrations could reach at least twice the $K_m$ values. Preliminary experiments did not show significant perturbation of renin activity in the presence of DMSO (2 to 10%). The kinetic parameters were calculated according to Wilkinson (36) from the hydrolysis rates for at least six substrate concentrations.

### Optimal pH Determination

The buffers used were as follows: 100 mM formiate (pH < 4.0), 100 mM acetate (4.0 < pH < 5.5), and 100 mM phosphate (pH > 5.5). The fluorescent fragments of each substrate were quantified at different time intervals at each pH by the fluorimetric method described above, but also by HPLC in order to verify whether peptide bonds other than Leu–Val were cleaved at different pH values.

### Inhibition of Human Renin

Isopropyl(2*R*,3*R*)-3-[[*N*-[(2*R*)-3-(morpholinocarbonyl)-2-(1-naphthylmethyl)propyl]-L-histidyl]-amino]-4-cyclohexyl-2-hydroxybutyrate, a kind gift of Dr. Y. Kiso from Kyoto Pharmaceutical University, was assayed as an inhibitor of hydrolysis of compound **II** by human renin in 100 mM phosphate buffer (pH 6.0), at 37°C.

### Renin Concentration Measurement

The amount of Abz-Phe-His-Leu released from a 0.8 μM solution of Abz-Phe-His-Leu-Val-Ile-His-EDDnp by different renin concentrations, at 37°C for 2 h, was quantified by fluorimetric procedures.

## RESULTS AND DISCUSSION

Leu–Val is the only scissile bond in the pH range 3 to 7 present in all the intramolecularly quenched fluorogenic peptides synthesized in this work (Table 1), as demonstrated by HPLC of the products of their hydrolyses by purified human renin, by a Haas preparation of human renin (Fig. 1), and by a crude extract of human kidney powder. The substrates obtained were not hydrolyzed by pig renin nor by the acidic protease isolated during our renin purification from human kidney, which presented properties very similar to those of cathepsin D. Although we have observed that this enzyme releases angiotensin I from the equine angiotensinogen (1–14), its failure to hydrolyze the quenched fluorogenic substrates agrees with the report of Poe *et al.* (12), who demonstrated that human angiotensinogen (1–14) is resistant to hydrolysis by human liver cathepsin D.

The fluorescence emission ratio between the sub-

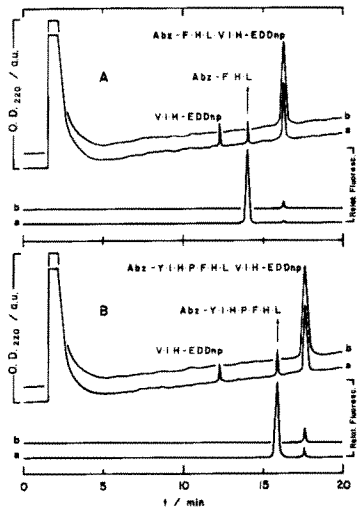


**FIG. 1.** RP-HPLC profile of compounds **I** (A) and compound **VI** (B) before (b) and after (a) incubation with human renin (Dr. Haas preparation) at pH 5.5.

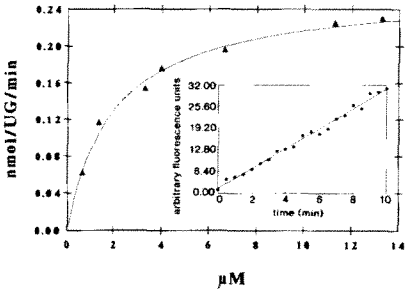


**FIG. 2.** Hydrolysis of Abz-His-Pro-Phe-His-Leu-Val-Ile-His-EDDnp by human renin at different substrate concentrations. Initial hydrolysis rates were determined as shown in the inset. The nonlinear least-squares fit to the Michaelis-Menten equation is shown by the solid line.

strate and products after total enzymatic hydrolysis for each peptide are shown in Table 1. The emission of fluorescence after total hydrolysis of the substrates increased 7- to 33-fold. This significant increase in fluorescence indicates an efficient quench effect of the 2,4-dinitrophenyl group over Abz fluorescence, possibly resulting from the proximity of the C- and N-extremities of the peptides. This is in agreement with our initial expectation that a $\beta$-turn folded conformation should be present in most of the substrates.

A typical profile of rate versus substrate concentration for the hydrolysis of Abz-His-Pro-Phe-His-Leu-Val-Ile-His-EDDnp is shown in Fig. 2. The increase in fluorescence per mole of cleaved substrate allowed accurate estimations of the initial reaction rates at concentrations lower than nanomolar. The pH dependence of the hydrolysis of compound **I** is a symmetrical bell-shaped curve centered around pH 5.5, but for compounds **II** to **VI** the curves were flat, with maximum enzymatic activities in the pH range 4 to 6.

The hydrolysis of compound **II** was inhibited by isopropyl(2*R*,3*R*)-3-[[*N*-[(2*R*)-3-(morpholinocarbonyl)-2-(1-naphthylmethyl)propyl]-L-histidyl]-amino]-4-cyclohexyl-2-hydroxybutyrate, a highly specific and potent human renin inhibitor described by Iizuka *et al.* (38). The $IC_{50}$ value, obtained at pH 6, was $7.8 \times 10^{-8}$ M, which is one order of magnitude higher than the $IC_{50}$ previously described at pH 7.4, using the natural human angiotensinogen as substrate (38). This difference could be related to the different pHs at which the $IC_{50}$ values were obtained since, for an efficient binding, the imidazole group of the inhibitor must be deprotonated (39). If the $pK_a$ value of this imidazole group is taken to be around 7 (40), at pH 6 ca. 10% of the imidazole should be deprotonated; therefore, our $IC_{50}$ value should be reduced by one order of magnitude and will be comparable to that of Iizuka *et al.* (38). This observation is in agreement with Maibaum and Rich (41), who related the high selectivity of potent renin inhibitors (in comparison to those of pepsin and cathepsin D) to the extent of protonation when histidine is located at the $P_2$ position, as is the case with the renin inhibitor that we assayed.

The hydrolysis of all the studied intramolecularly quenched fluorogenic substrates by human renin followed Michaelis-Menten kinetics, and the parameters for these reactions are shown in Table 2.

The $K_m$ values obtained for all quenched fluorogenic substrates do not differ significantly; therefore, their affinity to renin seems to be independent of their size and also of the substitution at positions $P_4$ and $P_5$. We have previously studied the hydrolysis of the N-terminal heptadecapeptide of human angiotensinogen (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Asn-Glu-Ser-Thr-$NH_2$), containing the putative glycosylation binding site, by the same human renin used in this work and the $K_m$ value obtained was 34 $\mu$M (34). $K_m$ values of about 25 $\mu$M were also obtained for the hydrolyses of the N-terminal trideca- and tetradecapeptides (3,42) of human angiotensinogen by human renin. A comparison of the structures of these peptides with those of fluorogenic substrates presented in this work indicates that the increase in affinity could be related to the presence of both Abz and EDDnp groups. As the sequence of the six C-terminal residues of all fluorogenic peptides (Table 1) are the same, it seems reasonable to attribute to Phe-His-Leu-Val-Ile-His-EDDnp a major role in the binding process of these substrates. On the other hand, the other residues at the N-terminal sites of the substrates have a significant influence on the catalytic process, since among compounds **I, II,** and **VI,** in the natural sequence, there is a progressive increase in the peptide length by two residues and the minimum $V_{max}$ value was observed with compound **II.**

TABLE 2

Kinetic Parameters (±SD) for the Hydrolysis of Intramolecularly Fluorogenic Substrates by Human Renin

| Substrate | $K_m$ ($\mu$M) | $V_{max}$ (nmol/GU · min) | $V_{max}/K_m$ (nmol/GU · min) · $\mu M^{-1}$ |
|---|---|---|---|
| **I** | 1.3 ± 0.2 | 17 ± 2 | 13.1 |
| **II** | 1.3 ± 0.2 | 0.71 ± 0.04 | 0.5 |
| **III** | 1.2 ± 0.2 | 0.71 ± 0.05 | 0.6 |
| **IV** | 1.6 ± 0.2 | 0.36 ± 0.02 | 0.2 |
| **V** | 2.0 ± 0.2 | 0.26 ± 0.01 | 0.1 |
| **VI** | 2.5 ± 0.3 | 37 ± 3 | 14.8 |

*Note.* Hydrolyses conditions: 37°C, 100 mM sodium acetate buffer (pH 5.5), with 0.1% gelatin and 10% DMSO.

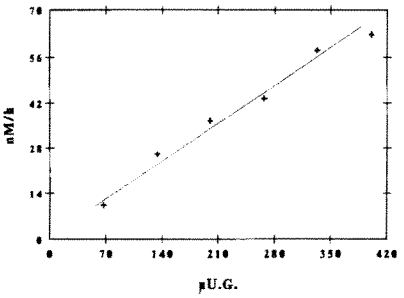


FIG. 3. Relationship between the increasing human renin concentration and Abz-Phe-His-Leu released from a 0.8 μM solution of Abz-Phe-His-Leu-Val-Ile-His-EDDnp, at 37°C, pH 5.5, for 2 h.

Therefore, compounds **I** and **VI** are fitted to the binding site in a manner that provides the best conditions for the catalytic hydrolysis of the Leu-Val bond.

Compounds **III**, **IV**, and **V** are analogs of compound **II**, in which Arg and Gly are substituted for His and Pro, respectively. These modifications did not improve the hydrolysis of the resulting substrates, although they increased the fluorescence quenching (compare $F_i/F_1$ values of Table 1) and the presence of Arg enhanced the solubility in water.

The specificity of our substrates to human renin seems to be related more to the presence of Ile-His at sites $P'_2$-$P'_3$ than to the Leu-Val at $P_1$-$P'_1$, since Pro-His-Pro-Phe-His-Phe-Phe-Ile-His-D-Lys is a substrate for human renin but its analog, Pro-His-Pro-Phe-His-Phe-Phe-Val-Tyr-D-Lys is not (37).

The sensitivity of renin assays with intramolecularly quenched fluorogenic substrate was examined with compound **I**. As indicated in Fig. 3, within a reaction time of 2 h, compound **I** was able to detect less than 70 μGU/ml of active human renin. The sensitivity of this quenched fluorogenic substrate is significantly higher than those described by Murakami *et al.* (11) for Arg-Pro-Phe-His-Leu-Leu-Val-Tyr-ACM or its succinyl derivative; however, it is ca. 30 times less sensitive than the radioimmunoassay of Haber *et al.* (6), which we performed with the N-terminal heptadecapeptide of human angiotensinogen using the same human renin employed in the present work (34).

In our preliminary experiments the potential use of the described substrates, particularly compound **I**, to evaluate the plasma renin concentration is very promising; however, it is necessary to reduce the background fluorescence of plasma in order to increase the sensitivity of the assays. Although the $K_m$ values of hydrolysis of our quenched fluorogenic peptides are on the micromolar order, it is necessary to enhance their solubility in water.

In conclusion, we described a series of intramolecularly quenched fluorogenic substrates with high specificity for human renin and with reasonable sensitivity that allow a continuous fluorimetric assay for this endopeptidase.

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Exhibit G

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# BACE1- and BACE2-expressing Human Cells

## CHARACTERIZATION OF β-AMYLOID PRECURSOR PROTEIN-DERIVED CATABOLITES, DESIGN OF A NOVEL FLUORIMETRIC ASSAY, AND IDENTIFICATION OF NEW *IN VITRO* INHIBITORS*

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**David Andrau‡, Cécile Dumanchin-Njock‡§, Erwan Ayral¶‖, Jean Vizzavona‖, Mike Farzan**, Michel Boisbrun¶, Pierre Fulcrand¶, Jean-François Hernandez¶, Jean Martinez¶, Solveig Lefranc-Jullien‡ ‡‡, and Frédéric Checler‡§§**

*From the ‡Institut de Pharmacologie Moléculaire et Cellulaire of Centre National de la Recherche Scientifique, UMR6097, 06560 Valbonne, France, the ¶Laboratoire des Amino Acides, Peptides et Protéines, CNRS UMR5810, 34093 Montpellier, France, and **Harvard Medical School, Boston, Massachusetts 02115*

**We have set up stably transfected HEK293 cells overexpressing the β-secretases BACE1 and BACE2 either alone or in combination with wild-type β-amyloid precursor protein (βAPP). The characterization of the βAPP-derived catabolites indicates that cells expressing BACEs produce less genuine Aβ1–40/42 but higher amounts of secreted sAPPβ and N-terminal-truncated Aβ species. This was accompanied by a concomitant modulation of the C-terminal counterpart products C89 and C79 for BACE1 and BACE2, respectively. These cells were used to set up a novel BACE assay based on two quenched fluorimetric substrates mimicking the wild-type (JMV2235) and Swedish-mutated (JMV2236) βAPP sequences targeted by BACE activities. We show that BACEs activities are enhanced by the Swedish mutation and maximal at pH 4.5. The specificity of this double assay for genuine β-secretase activity was demonstrated by means of cathepsin D, a "false positive" BACE candidate. Thus, cathepsin D was unable to cleave preferentially the JMV2236-mutated substrate. The selectivity of the assay was also emphasized by the lack of JMV cleavage triggered by other "secretases" candidates such as ADAM10 (A disintegrin and metalloprotease 10), tumor necrosis α-converting enzyme, and presenilins 1 and 2. Finally, the assay was used to screen for putative *in vitro* BACE inhibitors. We identified a series of statine-derived sequences that dose-dependently inhibited BACE1 and BACE2 activities with $IC_{50}$ in the micromolar range, some of which displaying selectivity for either BACE1 or BACE2.**

Alzheimer's disease is characterized by the cortical and subcortical accumulation of proteinaceous deposits called senile plaques (1). The main constituent of these aggregates is referred to as amyloid β peptide (Aβ).[1] Aβ is generated from the β amyloid precursor protein (βAPP) by the subsequent attacks by β- and γ-secretases, which liberate the N- and C-terminal moieties, respectively (2). It is now clear that under this generic terminology, "Aβ" gathers a series of distinct "Aβ-related species," some of them truncated at their β-secretase-derived N terminus (3). Although the nature of the γ-secretase is still discussed (4, 5), the identity of the β-secretase candidate is more consensual.

BACE1 (β-site APP-cleaving enzyme) is an aspartyl protease recently identified by several groups (6–9), which displays all the properties expected from a genuine β-secretase candidate (for review, see Refs. 10 and 11). Thus, BACE1 is an acidic protease mainly localized in the Golgi apparatus and in endosomal compartments and exhibits a lumenal active site fitting well with the lumenal cleavage of βAPP occurring in these acidic compartments where Aβ had been detected. The overexpression of BACE1 leads to increased recovery of Aβ-related fragments, mainly 1–40 and 11–40 that are also observed when recombinant BACE is incubated with βAPP (7, 9). The opposite phenotype is observed when BACE is down-regulated by an antisense approach (7, 9). BACE2 is a parent protease (12) that seems to contribute poorly to neuronal Aβ production. However, BACE2 could be important in Down syndrome pathology because the enzyme is encoded by chromosome 21 (13, 14) and elevated BACE2 expression is observed in trisomic brains (15).

BACE1 not only behaves as a β-secretase, *in vitro*, but likely corresponds to the main contributor of the β-secretase pathway, *in vivo*. Thus, it was evidenced that the invalidation of the *BACE* gene led to abolishment of Aβ production in knockout mice (16) and neurons (17). That this deletion appeared totally innocuous (18) makes BACE likely the most interesting target of an Aβ-directed AD therapy. Here we fully characterize the βAPP-derived catabolites generated by BACE1 and BACE2-

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§ Supported by the Fondation Alzheimer. Present address: INSERM EMI 9906, Rouen, France.

‖ Present address: Centre Medical Universitaire, Département de Chimie Médicinale, Genève, Switzerland.

‡‡ Supported by Aventis Pharma.

§§ To whom correspondence should be addressed: Institut de Pharmacologie Moléculaire et Cellulaire, UMR6097 du CNRS, 660 Route des Lucioles, Sophia Antipolis, 06560 Valbonne, France. Tel.: 33-493957760; Fax: 33-493957708; E-mail: checler@ipmc.cnrs.fr.

[1] The abbreviations used are: Aβ, amyloid β peptide; βAPP, β-amyloid precursor protein; Abz, *p*-aminobenzoic acid; tBu, *tertio*-butyl; EDDnp, *N*-(2,4-dinitrophenyl)ethylenediamine; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; HBTU, *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HPLC, high pressure liquid chromatography; Boc, *tert*-butyloxycarbonyl; *Sta*, statine; *Norsta*, norstatine; *AHPPA*, (3*S*,4*S*)-4-amino-3-hydroxy-5-phenyl-pentanoic acid; *ACHPA*, (3*S*,4*S*)-4-amino-5-cyclohexyl-3-hydroxy-pentanoic acid; *Qui*, quinolein-2-carboxylic acid; TACE, tumor necrosis α-converting enzyme; CS, commercial substrate; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; Bz, benzoyl.

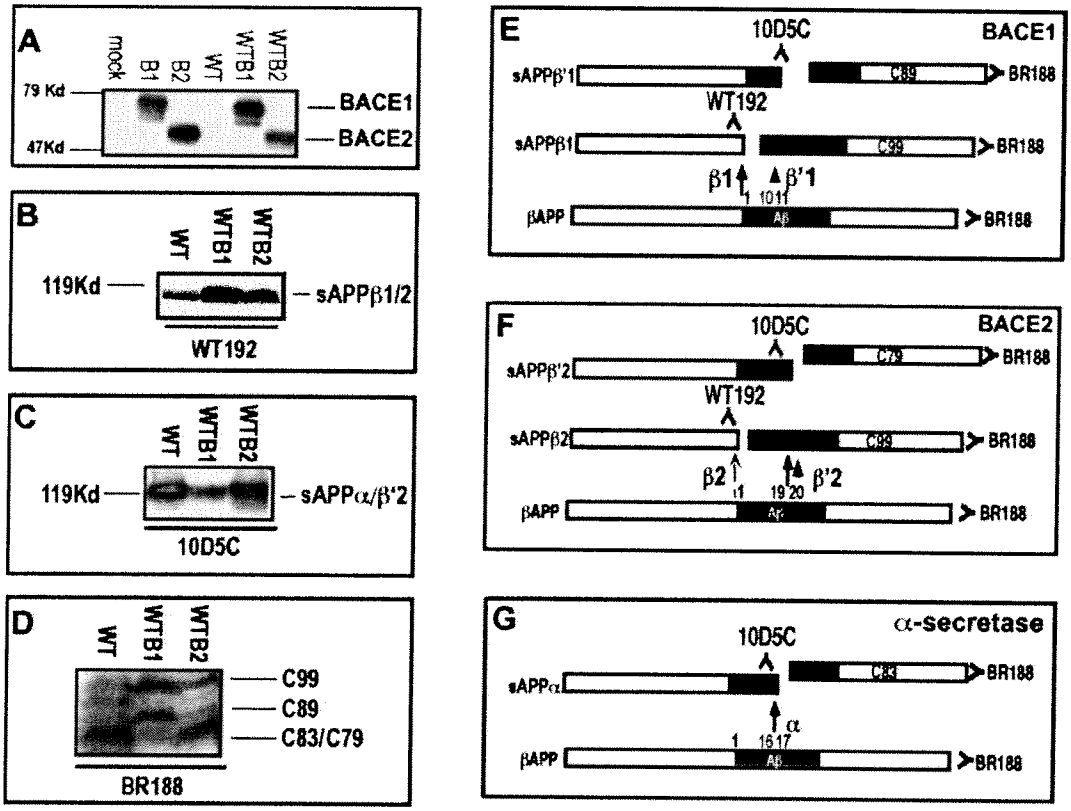



FIG. 1. **Characterization of secreted βAPP-derived products and their C-terminal counterparts generated by BACE1- and BACE2-expressing cells.** HEK293 cells overexpressing 1D4-tagged BACE1 or BACE2 alone (*B1* and *B2*, respectively) or in combination with wild-type βAPP (*WTB1* and *WTB2*) have been obtained as described under "Experimental Procedures" and monitored by means of anti-1D4 antibodies (*panel A*). sAPPβ1/2 derived from BACE1 (*panel E*) and BACE2 (*panel F*) are identical and are monitored by means of WT192 (*panel B*) as described under "Experimental Procedures." sAPPα and sAPPβ'2 generated by α-secretase (*panel G*) and BACE2 (*panel F*), respectively, were monitored by means of 10D5C (*panel C*) as described under "Experimental Procedures." Intracellular C-terminal counterparts derived from the above cleavages were monitored in the indicated cell lysates with BR188 (*panel D*) as described under "Experimental Procedures."

expressing cells. Furthermore, we describe a novel fluorimetric assay and new *in vitro* inhibitors of potential interest to further characterize BACE1 and BACE2 properties.

## EXPERIMENTAL PROCEDURES

*Chemical Reagents*—Fmoc-amino acids, HBTU, and the resin were purchased from Senn Chemicals (Gentilly, France). The reagents and solvents for solid-phase peptide synthesis were obtained from Acros (Noisy-le-Grand, France) or from SDS (Peypin, France). All other chemicals were of the purest grade available.

*Synthesis of Peptides and Inhibitors*—The intramolecularly quenched fluorogenic peptidic substrates Abz-Val-Lys-Met-Asp-Ala-Glu-EDDnp (JMV2235) and Abz-Val-Asn-Leu-Asp-Ala-Glu-EDDnp (JMV2236) contain the *ortho*-aminobenzoyl (Abz)/dinitrophenyl groups as the donor/acceptor pair (19). They were synthesized essentially by solid phase methods (20), starting from the C-terminal residue Glu-EDDnp. The latter was first assembled in solution by coupling Fmoc-Glu(γ-tBu)-OH to EDDnp using HBTU as the coupling agent, in the presence of diisopropylethylamine. The resulting Fmoc-Glu(γ-tBu)-EDDnp was treated by trifluoroacetic acid to free the γ carboxylate group that was subsequently attached to the linker of a Wang resin by the symmetric anhydride method. After manual assembling of the substrates using the solid phase Fmoc strategy and HBTU as the coupling agent, cleavage of the peptides from the resin and simultaneous deprotection of side chains were carried out by treatment with a solution containing trifluoroacetic acid, thioanisole, water, phenol (8.5/0.5/0.5/0.5) for 2 h at room temperature. The final deprotected peptides were purified by reverse-phase chromatography on a C18 column (Deltapack Waters, 40 × 100 mm) by means of a linear gradient of 20–35% acetonitrile in 0.1% aqueous trifluoroacetic acid over 30 min (flow rate 50 ml/min), and their purity and identity were assessed by reverse-phase HPLC and electrospray mass spectrometry (Abz-VKMDAE-EDDnp: experimental mass 1018.7 ± 0.2, calculated mass 1018.4; Abz-VNLDAE-EDDnp: experimental mass 986.6 ± 0.2, calculated mass 986.4).

All peptidic inhibitors were amidated at their C-terminal end and blocked at their N terminus, either by an acetyl or by a Boc group (excepted the Qui-containing compounds), to protect them from exopeptidasic attacks. The peptide backbone of the compounds was stepwise assembled by classical methods, using Boc as the α-amino protecting group and benzotriazol-1-yloxy tris(dimethylamino)phosphonium hexafluorophosphate as the coupling reagent, either in homogeneous phase or on solid phase with methylbenzhydrylamine resin, necessitating a final HF cleavage procedure. Published protocols were followed for the formation of the peptide bond isostere moieties, reduced amide bond (Leu-Ψ($CH_2NH$)-Asp in JMV963) (21, 22), norstatine (*Norsta*-containing compounds) (23, 24), and statine and analogs (*Sta*-, *AHPPA*-, *ACHPA*-containing compounds) (25, 26). All synthetic inhibitors were purified on C18 reverse-phase HPLC, and their purity and identity were assessed by reverse-phase HPLC and electrospray mass spectrometry.

*HEK293 Culture and Stable Transfection*—HEK293 and stably transfected HEK293 cells overexpressing wtβAPP751 (WT) and swβAPP751 (SW) (27) were stably transfected with DAC30 (according to the manufacturer's instruction Eurogentec) containing 2 μg of pcDNA3 vector encoding either 1D4-tagged BACE1 (WTB1) or BACE2 (WTB2) and zeocin resistance. 1D4 is a 10-amino acid C-terminal tag derived from bovine rhodopsin (28). Medium was replaced 48 h after transfection with selective medium containing neomycin and/or zeonin (1 g/liter). Transfectants were screened by 10% SDS-PAGE gels analysis and Western blotting (see below). Positive clones overexpress 75- and 50-kDa immunoreactive proteins, respectively, corresponding to BACE1 and BACE2 (28). Tumor necrosis α-converting enzyme (TACE), A disintegrin and metalloprotease 10 (ADAM10), and presenilin 1 and presenilin 2-expressing cells were previously described (29–31).

*Western Blot Analyses*—HEK293 cells were scraped and lysed in

RIPA 1× buffer (10 mM Tris, pH 7.5, containing 150 mM NaCl, 5 mM EDTA, 0.1% deoxycholate, and 1% Nonidet P-40). Equal quantity of proteins were separated on a 10% SDS-PAGE gel for the detection of BACE1 and BACE2 proteins and transferred to Hybond-C (Amersham Biosciences) membranes. After transfer, membranes were blocked with 5% nonfat milk and incubated overnight with the primary antibody anti-1D4 (1:1000) (mouse monoclonal antibody donated by Scott Waniger at the National Cell Culture Center, National Cell Culture Center, Minneapolis, MN). Immunological complexes were revealed with an anti-mouse peroxidase (Amersham Biosciences) antibody followed by enhanced chemiluminescence (Amersham Biosciences). All protein concentrations were determined by the Bradford (32) procedure as described.

*Measurements of Total Aβ*—Stably transfected WT, WTB1, WTB2, and SW HEK293 cells (see above) were incubated for 7 h in the presence of phosphoramidon (1 μM) (Sigma). Media were collected, diluted in 1/10 RIPA 10× buffer (10 mM Tris, pH 7.5, containing 150 mM NaCl, 5 mM EDTA, 0.1% SDS, and 1% Nonidet P-40), and incubated overnight with a 200-fold dilution of FCA18 (total Aβ) as described (33). After further incubation for 3 h with protein A-Sepharose (Zymed Laboratories Inc.) and centrifugation, pelleted proteins were submitted to 16.5% Tris/Tricine gels and then Western blotted on Hybond C membranes (Amersham Biosciences) for 45 min. Nitrocellulose sheets were heated in boiling phosphate-buffered saline for 5 min and capped with 5% skim milk in phosphate-buffered saline containing 0.05% Tween 20 for 1 h. Membranes were then incubated overnight with WO2 antibody (ABETA GmbH, Heidelberg, Germany) specific for the 5–8 N-terminal region of Aβ (34)) and detected by enhanced chemiluminescence.

*Measurements of sAPPβ, sAPPα, and C-terminal Products*—WT, WTB1, and WTB2 cells were allowed to secrete for 7 h as above and then 15 (sAPPα/β′2) or 50 μl (sAPPβ1/2) of secretate were loaded on 8% SDS-PAGE and Western blotted with 10D5C or WT192 (kind gift from Dr. D. Schenk, Elan Pharmaceuticals), respectively. Immunological complexes were revealed with the adequate IgG coupled to peroxidase. C-terminal products were analyzed from the same cell lysates and were separated on large Tris/Tricine 16.5% gels, Western blotted, and probed with BR188 as described (35).

*Fluorimetric Assay*—Different cell types were lysed, in 10 mM Tris, pH 7, and then various amounts of total homogenate proteins were preincubated for 10 min with a commercial β-secretase inhibitor (KTEEISEVN-(*Sta*)-VAEF-OH, Enzyme System Products, Livermore, CA) or with distinct JMV inhibitors (10 μM) in 96-well plates. The JMV2235, JMV2236, or a β-secretase commercial substrate (Mca-SEVNLDAEFRK-(dinitrophenyl)-RR-$NH_2$, R & D Systems) were then added (10 μM) and incubated for various times at 37 °C. At the end of the incubation, fluorescence was recorded at 320 and 420 nm as excitation and emission wavelengths, respectively. When the effect of pH was monitored, lysed cells were pelleted, then resuspended in 25 mM $Na^+$-acetate/MES/Tris/TES adjusted at pH values ranging from 2 to 10 and then incubations were performed as above. Controls include membrane proteins or substrate alone and background fluorescence was subtracted to recorded BACE activities. This fluorescence was very low and did not change with the time of incubation at 37 °C. Cathepsin D activity (Sigma, 0.5 μg) was assayed as above with JMV2235, JMV2336, or with the commercial substrate.

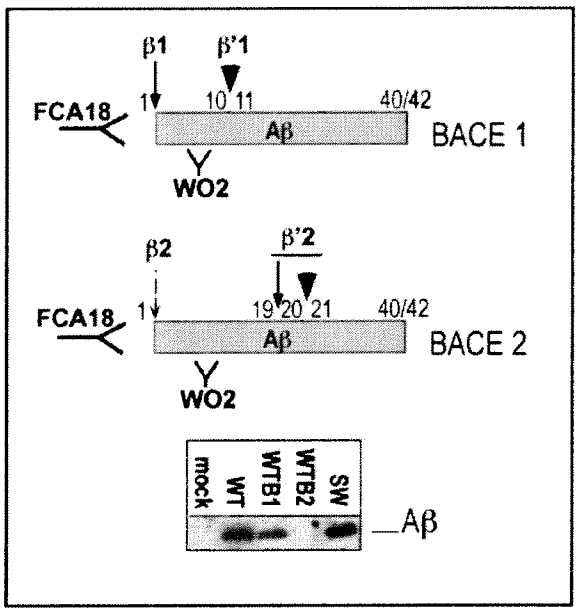



FIG. 2. **Characterization of genuine Aβ secreted by BACE1- and BACE2-expressing cells.** Mock-transfected HEK293 cells or cells expressing either Swedish-mutated βAPP (*SW*), wild-type βAPP alone (*WT*), or in combination with BACE1 or BACE2 (*WTB1* and *WTB2*, respectively) were allowed to secrete Aβ for 7 h at 37 °C and then genuine Aβ was immunoprecipitated with FCA18 (which recognizes only the Asp-1 residue liberated by β1 and β2 cleavages). Aβ was then monitored after Tris/Tricine gel analysis and Western blot with W02 as described under "Experimental Procedures."

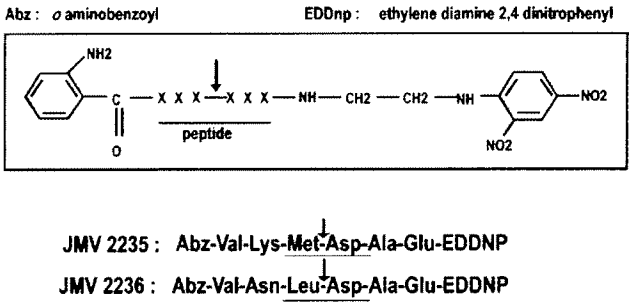



FIG. 3. **Structure of the JMV2235 and JVMV2236 quenched fluorimetric substrates.**

## RESULTS

*Characterization of Secreted and Intracellular βAPP-derived Products Generated by BACE1 and BACE2-expressing Human Cells*—We have set up stably transfected HEK293 human cells overexpressing 1D4-tagged BACE1 and BACE2 with expected molecular weights (Fig. 1*A*), either alone (called B1 and B2) or in combination with wild-type βAPP (referred to as WTB1 and WTB2). We have used WT192 monoclonal antibodies that recognize the two last amino acids of the secreted products derived from the canonical β-secretase cleavages taking place at the N terminus of the Asp-1 residue of Aβ (see β1 and β2 sites in Fig. 1, *E* and *F*). sAPPβ1 and sAPPβ2 generated by BACE1 and BACE2 were identical (Fig. 1, *E* and *F*) and theoretically both were recognized by WT192. As expected, secreted WT192-immunoreactive products were recovered in higher amounts in both BACE1- and BACE2-expressing cells than in WT cells (Fig. 1*B*). Additional β′1- and β′2-derived cleavages targeted by BACE1 and BACE2, respectively, occur inside the Aβ sequence and theoretically liberate sAPPβ′1- and sAPPβ′2-secreted fragments (see Fig. 1, *E* and *F*). sAPPβ′2, as well as sAPPα, the physiological α-secretase-derived APP fragment, retain a 10D5C epitope that was disrupted by β′1 cleavage (see Fig. 1, *E* and *F*). As expected, 10D5C-positive fragments secreted by WT and WTB2 cells (corresponding to sAPPα/β′2) were recovered in much higher amounts than with WTB1 cells (Fig. 1*C*).

The C-terminal counterparts of the above products were probed in cell lysates using BR188, a polyclonal antibody that interacts with the C terminus of all fragments (Fig. 1, *E–G*). As expected, β1- and β2-derived cleavages (Fig. 1, *E* and *F*) increase C99 in WTB1 and WTB2 (Fig. 1*D*), in agreement with higher production of their N-terminal counterpart sAPPβ1/2 (Fig. 1*B*). The nature of C99 was confirmed by means of FCA18 (36), a polyclonal antibody that specifically interacts with the free Asp-1 residue of Aβ and C99 (not shown). A fragment of lower molecular weight was specifically recovered with WTB1 cells (Fig. 1*D*). Although not definitively identified, this product likely corresponds to C89, the β′1-derived specific product generated by WTB1 cells (Fig. 1, *D* and *E*). Finally, a low molecular

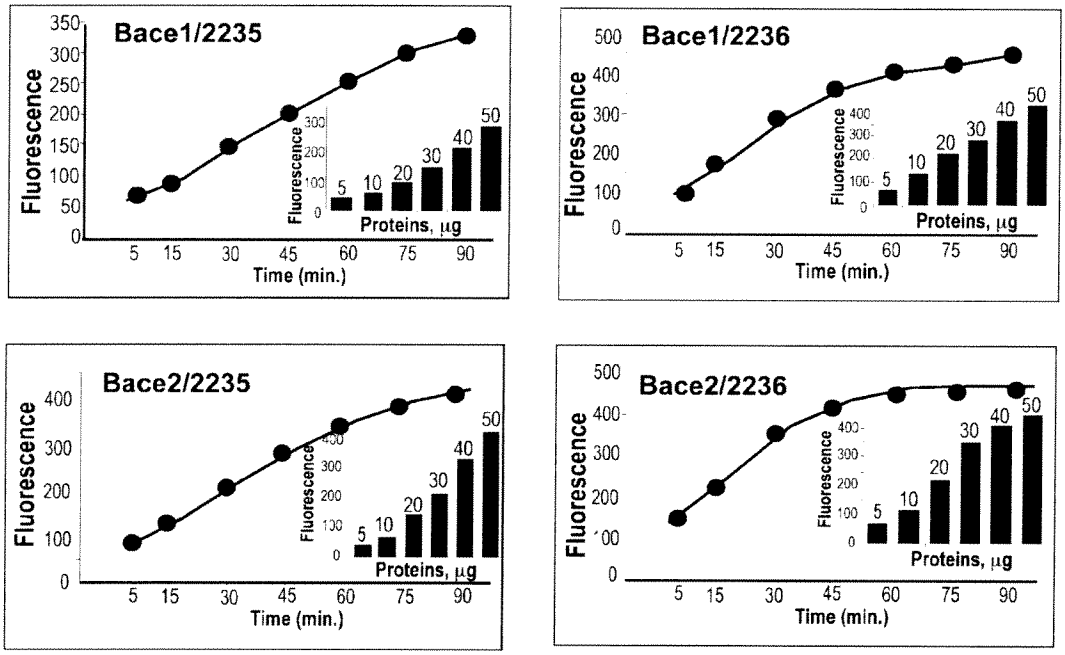



FIG. 4. **Kinetic analyses of JMV2235 and JMV2236 hydrolysis by BACE1- and BACE2-expressing cells.** BACE1- (*upper panels*) and BACE2- (*lower panels*) expressing cells (30 μg of proteins) were assayed for their JMV2235- (*left panels*) or JMV2236- (*right panels*) hydrolyzing activities for the times indicated at pH 4.5 as described under "Experimental Procedures." *Insets*, activities were measured for 30 min at pH 4.5 with the indicated varying amounts of proteins. Fluorescence was recorded at 320 and 420 nm as excitation and emission wavelengths, respectively, as described under "Experimental Procedures."

weight product present in BACE2 but not BACE1 cells could derive from the β′2 cleavage (Fig. 1*F*). This product tentatively ascribed to C79 co-migrates with the C83 major product generated by α-secretase in WT cells (Fig. 1, *D* and *G*). C89- and C79-like products were FCA18-negative (not shown), confirming that the fragments lack the intact N terminus present in C99.

*Characterization of Genuine Aβ in BACE1- and BACE2-expressing HEK293 Cells*—The use of FCA18 was proved useful to monitor secreted genuine Aβ. Thus, this antibody only recognizes free Asp-1 residue of Aβ or C99 (Fig. 2) because acetylation or removal of this aspartyl residue abolishes recognition by FCA18 (36). Therefore, FCA18 does not label N-terminal truncated Aβ-related species. As expected, the overexpression of wild-type βAPP increases Aβ1–40/42 (compare mock and WT in Fig. 2). Interestingly, as previously described (27, 37–39) the introduction of the Swedish mutation triggers increased Aβ secretion (compare WT and SW). Both WTB1 and WTB2 cells secrete less Aβ than WT cells (Fig. 2), indicating that the β′1 and β′2 cleavages are likely more efficient than the β1 and β2 breakdowns elicited by BACE1 and BACE2 (see Fig. 2), respectively. It is noteworthy that while "complete" Aβ is still detectable in BACE1-expressing cells, it was not recovered in WTB2. The fact that sAPPβ was augmented in BACE2 (Fig. 1*B*) suggests that the β2 cleavage before Asp-1 of Aβ sequence indeed occurs. However, the relatively faint augmentation of the C-terminal counterpart C99 and the accumulation of C79 (Fig. 1*D*) suggests that BACE2-elicited β′2 cleavage was particularly efficient and could take place using C99 as a substrate, thereby explaining the low recovery of "full-length Aβ" observed with these cells (Fig. 2).

*Design of a Novel BACE Fluorimetric Assay*—Several studies have indicated that the Swedish mutation responsible for a familial form of Alzheimer's disease leads to increased production of Aβ via an exacerbation of the β-secretase-derived cleavage (37–39). We have set up a new assay based on the cleavage of quenched fluorimetric substrates mimicking the wild-type (JMV2235) and Swedish-mutated (JMV2236) sequences (Fig. 3) targeted by β-secretase (s) with the assumption that a good assay should be reflected by a favored hydrolysis of mutated JMV2236.

At acidic pH, B1 and B2 cell extracts (see Fig. 1) hydrolyze both JMV2235 and JMV2236 in a time- and dose-dependent manner (Fig. 4). It should be noted that kinetic analyses indicate that activities recovered with JMV2236 plateaued at the same fluorescence value (Fig. 4). This could be because of either depletion of available substrate or, alternatively, to an inhibitory effect by one of the products of the reaction. The latter hypothesis was ruled out by the fact that the rates of hydrolyzes of JMV2235 by B1 and B2 cells were not affected by 100 μM Bz-VNL, Bz-VKL, Bz-VNL-$NH_2$, and Bz-VKL-$NH_2$. These N-terminal degradation products lack the N-terminal fluorescent moiety and were either free or amidated at the C terminus (to prevent it from putative carboxypeptidase attack (not shown). In agreement with a substrate extinction, standard fluorescence observed with synthetic Abz-VNL (10 μM) corresponds to the value observed at plateau when 10 μM substrate is used, indicating that this fluorescence indeed corresponds to 100% hydrolysis of the substrate (not shown).

We used a β-secretase commercial inhibitor to further validate our assay. Interestingly, mock-transfected cells exhibit faint JMV2235- and JMV2236-hydrolyzing activities that remained insensitive to the inhibitor (Fig. 5, *A* and *B*), indicating that endogenous β-secretase activity was low in HEK293 cells. The JMV2235- and JMV2236-hydrolyzing activities of B1 cells returned to the mock-transfected cell value in the presence of the inhibitor (Fig. 5, *A* and *B*), indicating that most of the fluorescence recorded was indeed because of BACE1 in B1 cells. This conclusion also stands for BACE2-expressing cells (Fig. 5*C*) although the extent of inhibition was slightly lower (69 *versus* 97% of inhibition for B2 and B1 cells, respectively, see Fig. 6*B*). Interestingly, in B1 cells, hydrolysis of JMV2236-

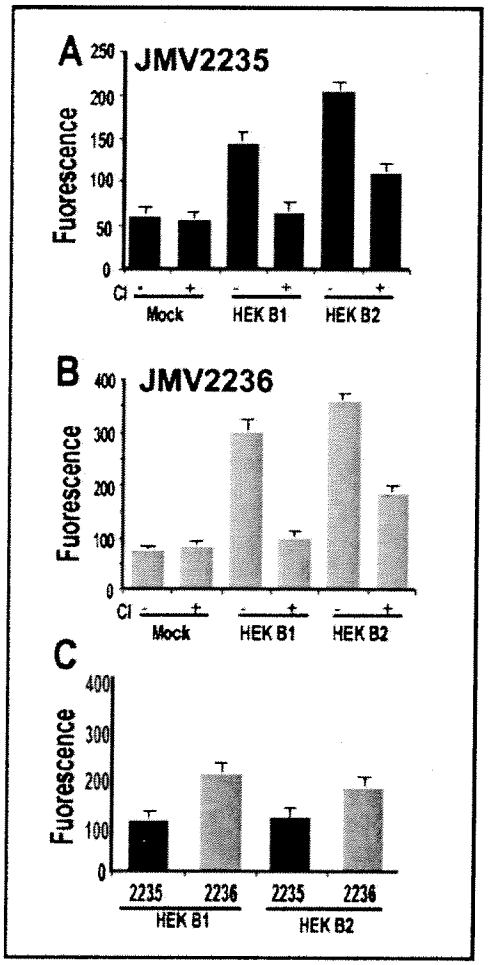
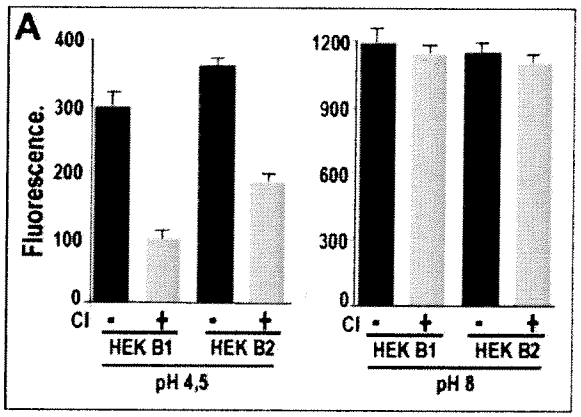



FIG. 5. **Comparative analysis of JMV2235 and JMV2236 hydrolysis by BACE1 and BACE2 activities: effect of a β-secretase commercial inhibitor.** Mock, BACE1- (*HEKB1*), or BACE2- (*HEKB2*) expressing cell proteins (30 μg) were incubated with 10 μM JMV2235 (*A*) or JMV2236 (*B*) for 30 min at pH 4.5 in the absence (−) or in the presence (+) of a commercial β-secretase inhibitor (CI, 10 μM), and then the activity was fluorimetrically recorded as described under "Experimental Procedures." *Bars* are the mean ± S.E. of eight determinations. *Panel C* compares the JMV2235- and JMV2236-hydrolyzing activities displayed by HEKB1 and HEKB2 cells.




| B | HEK B1 | | HEK B2 | |
|---|---|---|---|---|
| V (F/h/mg) | pH 4,5 | pH 8 | pH 4,5 | pH 8 |
| Control | 19998 | 79992 | 24064 | 78867 |
| + Inhibitor | 6599 | 76659 | 12332 | 75210 |
| % Inh° | 97 | 4 | 69 | 5 |

FIG. 6. **Inhibitor-sensitive JMV2236 hydrolysis by BACE1- and BACE2-expressing cells is pH-dependent.** BACE1- (*HEKB1*) or BACE2- (*HEKB2*) expressing cell proteins (30 μg) were incubated with 10 μM JMV2236 (*A*) for 30 min at pH 4.5 or 8, in the absence (−) or presence (+) of a commercial β-secretase inhibitor (*CI*, 10 μM), then the activity was fluorimetrically recorded as described under "Experimental Procedures." *Bars* are the mean ± S.E. of eight determinations. Specific activities in *B* correspond to initial velocity measurements of the JMV2236-hydrolyzing activity obtained with or without inhibitor. The % of inhibition was calculated after subtraction of the CI-insensitive activity obtained in mock-transfected cells (see "Results"). A fluorescence of 700 corresponds to 1 nmol of Abz-VNL-OH liberated.

mutated substrate is twice as efficient as that observed for the non-mutated analog (Fig. 5*C*).

Another clue for stating that our novel assay was β-secretase-specific was the strong pH requirement observed. We have carried out the assay at pH ranging from 2 to 10 (in 25 mM $Na^+$-acetate/MES/Tris/TES to avoid any intrinsic influence other than the pH). The activity was sharply maximal at pH 4–4.5 for JMV2236- (Fig. 6*A*) and JMV2235- (not shown) hydrolyzing activities in both B1 and B2 cells. It was interesting to note that at pH 8, very high JMV2236-cleaving activities were displayed by B1 and B2 calls but that, unlike at pH 4.5, remains totally insensitive to the β-secretase commercial inhibitor (Fig. 6, *A* and *B*). This further indicates that our assay allows selective dosage of β-secretase only at pH relevant for BACE biological activity.

*Comparison of JMV-based Assay with a Commercial β-Secretase Assay by Use of Cathepsin D*—BACE1 and BACE2 hydrolyze another commercial fluorimetric substrate (CS) with identical pH (Fig. 7*A*) and inhibitor-sensitive (Fig. 7, *A* and *C*) manners. At acidic pH, CS appeared even better cleaved by B1 and B2 cells than JMV2235 and JMV2236 substrates (Fig. 7*B*). We took advantage of the description of cathepsin D as an *in vitro* β-secretase-like activity (40, 41) to further compare the usefulness and accuracy of these two assays. Interestingly, although purified cathepsin D potently hydrolyzed CS and JMV2235, this enzyme was unable to hydrolyze the mutated substrate JMV2236 (Fig. 8, *A* and *B*). This shows that on the basis of the hydrolysis of CS or JMV2235 only, cathepsin D would have behaved as a good β-secretase candidate. The dual screening with JMV2235 and -2236 makes it fall in the category of the β-secretase "false positive" candidates. These data indicate the drastic improvement brought by our new assay for the monitoring of putative unknown β-secretase-like candidates and also confirm the fact that cathepsin D is not β-secretase. The selectivity of the assay was further emphasized by the inability of ADAM10, TACE, and presenilins 1 and 2, the α- and γ-secretases candidates to cleave JMV2236 (Fig. 9).

*Novel in Vitro Inhibitors of BACE1 and BACE2*—The above data suggested the use of JMV2236 as a good probe to screen for putative *in vitro* β-secretase inhibitors. We have studied the putative inhibitory effect of 26 peptidic sequences modified at the β-secretase site and harboring various N- and C-terminal lengths (Table I). Most of the molecules are displaying a statine-derived group, a non-cleavable residue that mimics the tetrahedral intermediate of catalysis by aspartyl proteases. The statine group can be replaced by an AHPPA ((3*S*,4*S*)-4-amino-3-hydroxy-5-phenyl-pentanoic acid) moiety without influencing the inhibitory activity toward both BACE1 and BACE2 (compare JMV1197 and JMV1200 in Table I and Fig. 10). JMV1195 and JMV1197 block BACE1 (Fig. 10*A*) and

BACE2 (Fig. 10*B*) with $IC_{50}$ values in the micromolar range (Fig. 10*C* and Table II), suggesting that shortening the N-terminal part of the inhibitor does not significantly alter its potency. The C-terminal length appears more important because shortening it by only one amino acid leads to an inactive inhibitor on BACE2 and less potent agent against BACE1 (compare JMV1196 and JMV1197; Fig. 10, *A* and *B*). It is noteworthy that this screening led to the identification of molecules that appear to discriminate between BACE1 and BACE2. This appears to be the case for JMV1321 which is more potent on BACE2 than on BACE1 whereas the contrary is true for JMV1196 (Fig. 10, *A* and *B*).

## DISCUSSION

Although the etiology of Alzheimer's disease is not formally known, it is difficult to consider the overproduction of the amyloid β peptide (Aβ) as an innocuous and inert event in the progression of the disease. Causative or not, Aβ is the most obvious biochemical common denominator between sporadic and familial forms of this disease. Thus, the mutations triggering the genetic forms of Alzheimer all lead to an acceleration of the disease progression that, if not directly because of a modulation of Aβ production, appears at least linked to it (for reviews, see Ref. 42–45). This so called "amyloid cascade" hypothesis (46) implies that the secretases that generate Aβ could be considered as major targets of therapeutic strategies aimed at slowing down the onset and progression of the disease.

Aβ is generated from a transmembrane precursor, the β-amyloid precursor protein, by subsequent attacks by β- and γ-secretases, which liberate the N- and C-terminal moieties of Aβ, respectively (for reviews, see Refs. 2 and 47–49)). The nature of the γ-secretase is still discussed (4, 5, 50, 51) and awaits definitive identification whereas the β-secretase function is more consensually ascribed to BACE1 (β-site APP cleaving enzyme also called memapsin 2 or Asp2, (6–9)). Both β- and γ-secretase inhibition could be theoretically seen as a means to prevent Aβ production but several apparently uncircumventable problems lead to the conclusion that γ-secretase is not an adequate target. Thus, inhibitors of the γ-secretase activity not only prevent Aβ production but also alter the processing of various proteins involved in vital functions at adulthood (52–56). Furthermore, preventing βAPP processing at the γ-secretase site increases the recovery of the highly toxic C99 product (3) that accumulates in AD brains (57).

At first sight, β-secretase appears as a much better target. First, it is noteworthy that BACE expression and activity are elevated in sporadic Alzheimer's disease brains, particularly in the cortical and hippocampal areas affected in the disease (58–60). Second, the abrogation of the *BACE1* gene totally abolishes the formation of Aβ-related species and C99 product in knockout neurons (17) and BACE1-deficient mice brain (16). Of most interest, mice devoid of BACE1 develop normally and have an unaltered phenotype (16, 18). The latter indicates that even if BACE1 specificity for βAPP is not exclusive, the other targeted substrates do not share essential functions or that another enzyme can complement for BACE-mediated proteolysis. This contrasts with presenilins-dependent γ-secretase-like cleavages, which when abolished, trigger lethality at the embryonic stage (61–63) and severe alterations at adulthood, particularly in thymocyte development (52, 64).

We have set up stable transfectants overexpressing BACE1. When expressed together with βAPP, we observed that the production of sAPPβ and C99, the two β-secretase-derived complementary products were increased, but to a much lesser extent for the latter. This is likely because of the subsequent cleavage of C99 inside the Aβ domain, leading to an N-terminal-truncated fragment as previously described (7, 65, 66). This agreed well with our observation that genuine Aβ, *i.e.* Aβ starting at the canonical Asp-1 residue, was drastically reduced after overexpression of BACE1. These features also stand and were even accentuated when studying cells overexpressing BACE2, the BACE1 parent protein. In this case, full-length Aβ appeared barely detectable in agreement with studies indicating that BACE2 mainly cleaved in the middle of the Aβ sequence, after the 19th and 20th residues (28, 67, 68), thereby

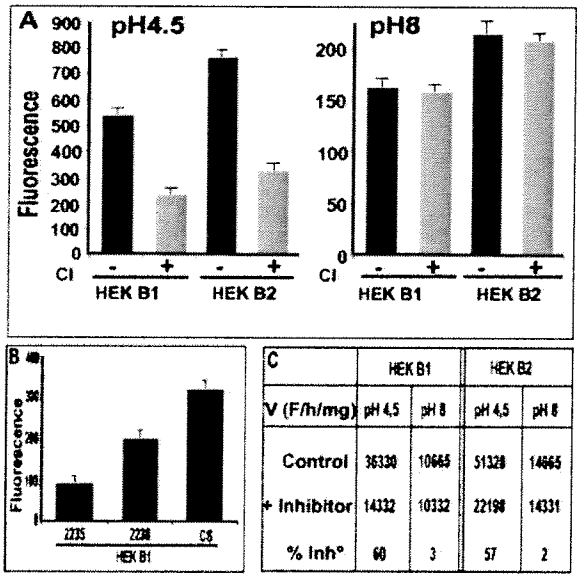



FIG. 7. **BACE1 and BACE2-hydrolyzing activity toward a β-secretase commercial substrate.** BACE1- (*HEKB1*) or BACE2- (*HEKB2*) expressing cell proteins (30 μg) were incubated with a commercial β-secretase substrate (10 μM) for 30 min at pH 4.5 or 8, in the absence (−) or presence (+) of a commercial β-secretase inhibitor (*CI*, 10 μM) and then the activity was fluorimetrically recorded as described under "Experimental Procedures." *Bars* are the mean ± S.E. of six (*C*) or eight (*A* and *B*) determinations.

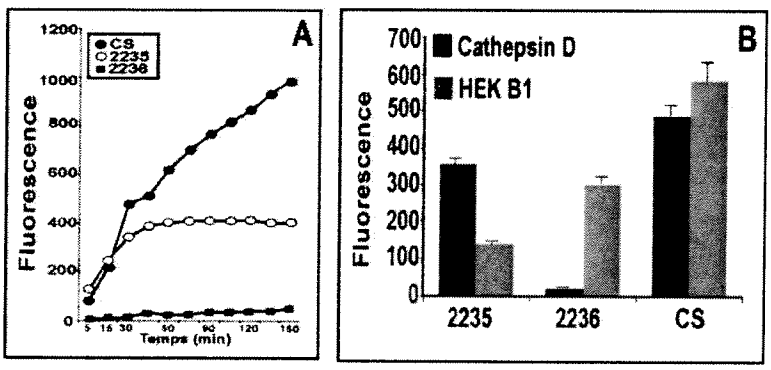



FIG. 8. **Hydrolysis of JMV2235, JMV2236, and the β-secretase commercial substrate by purified cathepsin D.** *A*, purified cathepsin D activity (0.5 μg) was assayed for the indicated time periods at pH 4.5 with 10 μM JMV2235, JMV2336, or commercial substrate (*CS*). In *B*, *bars* are the mean ± S.E. of six determinations and compare the fluorescence generated from the indicated substrates by cathepsin D and BACE1. A fluorescence of 700 corresponds to 1 nmol/30 min of Abz-VNL-OH liberated.

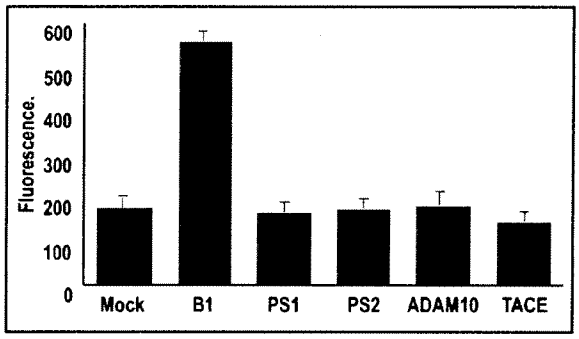



FIG. 9. **Hydrolysis of JMV2236 by several "secretase" candidates reveals selectivity for β-secretase.** Mock-transfected HEK293 cells or cells expressing BACE1 (*B1*), presenilin 1 (*PS1*), presenilin 2 (*PS2*), ADAM10 or TACE were obtained as described under "Experimental Procedures." Thirty μg of proteins were assayed for 30 min at pH 4.5 (B1 cells) or neutral pH (other cells) with 10 μM JMV2236 and then the activity was fluorimetrically recorded as described under "Experimental Procedures." *Bars* are the mean ± S.E. of eight determinations.

TABLE I
*Inhibitors nomenclature and structures*

| JMV | Inhibitors |
|---|---|
| 963 | Boc-Asn-Leu-ψ(*CH*$_2$*NH*)-Asp-Ala-NH$_2$ |
| 946 | Boc-Asn- *Sta* -Asp-Ala-NH$_2$ |
| 945 | Boc-Val-Asn- *Sta* -Ala-NH$_2$ |
| 931 | Boc-Asn- *Sta* -Ala-NH$_2$ |
| 947 | Boc-Asn- *Sta* -Ala-Glu-NH$_2$ |
| 1104 | Ac-Glu-Val-Asn- *Sta* -Ala-Glu-Phe-NH$_2$ |
| 1200 | Ac-Val-Asn- *Sta* -Ala-Glu-Phe-NH$_2$ |
| 1201 | Ac-Asn- *Sta* -Ala-Glu-Phe-NH$_2$ |
| 1105 | Ac-Val-Asn- *Sta* -Ala-Glu-NH$_2$ |
| 1242 | Ac-Glu-Val-Lys- *Sta* -Ala-Glu-Phe-NH$_2$ |
| 1251 | Qui-Val-Asn- *Sta* -Ala-NH$_2$ |
| 1250 | Qui-Asn- *Sta* -Ala-NH$_2$ |
| 1318 | Qui-Asn-*Norsta*-Asp-NH$_2$ (S,S) |
| 1317 | Qui-Asn-*Norsta*-Asp-NH$_2$ (S,R) |
| 1319 | Qui-Val-Asn-*Norsta*-Asp-NH$_2$ (S,S) |
| 1320 | Qui-Val-Asn-*Norsta*-Asp-NH$_2$ (S,R) |
| 1244 | Boc-Asn-*Norsta*-Ala-NH$_2$ (S,S) |
| 1245 | Boc-Asn-*Norsta*-Ala-NH$_2$ (S,R) |
| 1322 | Ac-Glu-Val-Asn-*Norsta*-Ala-Glu-Phe-NH$_2$ (S,S) |
| 1321 | Ac-Glu-Val-Asn-*Norsta*-Ala-Glu-Phe-NH$_2$ (S,R) |
| 1195 | Ac-Glu-Val-Asn-*AHPPA* -Ala-Glu-Phe-NH$_2$ |
| 1197 | Ac-Val-Asn-*AHPPA* -Ala-Glu-Phe-NH$_2$ |
| 1202 | Ac-Asn-*AHPPA* -Ala-Glu-Phe-NH$_2$ |
| 1196 | Ac-Val-Asn-*AHPPA* -Ala-Glu-NH$_2$ |
| 1243 | Ac-Glu-Val-Lys-*AHPPA* -Ala-Glu-Phe-NH$_2$ |
| 1300 | Ac-Glu-Val-Asn-*ACHPA* -Ala-Glu-Phe-NH$_2$ |

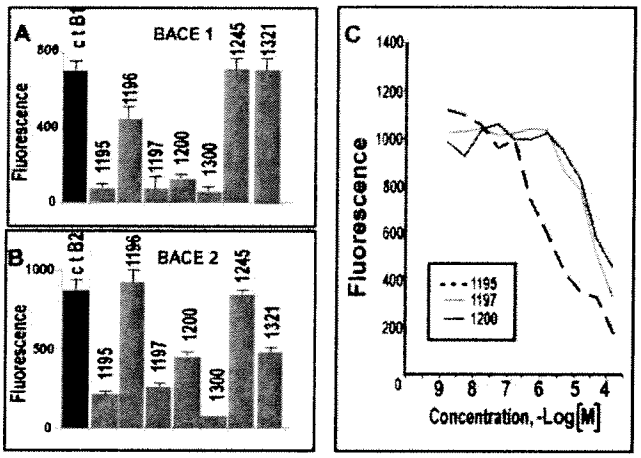



FIG. 10. **Effect of new inhibitors toward BACE1 and BACE2.** BACE1- (*A*) or BACE2- (*B*) expressing cell proteins (30 μg) were incubated with JMV2236 (10 μM) for 30 min at pH 4.5 in the presence of the indicated JMV inhibitors (10 μM) and then the activity was fluorimetrically recorded as described under "Experimental Procedures." *Bars* are the mean ± S.E. of four determinations. In *C*, a complete dose-response curve of the indicated JMV inhibitors obtained with BACE2-expressing cells is shown.

TABLE II
*$IC_{50}$ values of inhibitors on BACE1 and BACE2 activities*

| Inhibitor | $IC_{50}$ | |
|---|---|---|
| | B1 cells | B2 cells |
| Commercial inhibitor | 1 μM | 10 μM |
| JMV1195 | 3 μM | 1 μM |
| JMV1197 | 3 μM | 10 μM |
| JMV1200 | 3 μM | 10 μM |
| JMV1300 | 1 μM | 1 μM |

leading to N-terminal truncated products that escaped immunological detection in our conditions. In line with these data, N-terminal truncated C89 and C79 accumulated in BACE1- and BACE2-expressing cells, respectively.

We have set up a new β-secretase assay based on the hydrolysis of two quenched fluorimetric substrates, one of which harboring the Swedish mutation is thought to enhance β-secretase cleavage (see Introduction). The assay allows to monitor a time- and dose-dependent fluorescence specifically increased by BACE1 and BACE2 overexpression. More important, we establish that this activity was maximal at acidic pH and enhanced by the Swedish mutation, in agreement with the reported properties of β-secretase activity (for reviews, see Refs. 10, 11, and 69). It should be noted that the effect of the mutation on BACE activity appears weaker with these fluorigenic substrates than with βAPP itself. This is likely because of the fact that BACEs act better as protease rather than peptidases. This semantic discrimination implies that these proteases hydrolyze preferentially proteins rather than small peptides because the former likely fit better with a relatively larger recognition/catalytic pocket (70).

It is interesting to note that the signature of a genuine β-secretase activity is indeed revealed by the mutation-induced differential fluorescence monitored by this dual assay. Thus, cathepsin D, a protease with *in vitro*-like β-secretase activity (40, 41), indeed cleaves efficiently a commercial substrate mimicking the β-secretase-targeted sequence. However, our assay demonstrated that this protease did not behave as a good β-secretase candidate because it did not hydrolyze JMV2236, the fluorimetric substrate bearing the Swedish mutation. This dual assay therefore proved useful to monitor genuine β-secretase activities. In this context, it is interesting to note that recently, splice variants of BACE1 have been identified in human brain and pancreas (71, 72). Our assay should allow the monitoring of other putative BACE-like activities and help reveal yet unknown functions of these activities.

The most potential interest of our assay would be to design highly potent, bioavailable and metabolically stable inhibitors of BACE1. This is a real challenge because until now, the inhibitors designed are mostly peptide-based (for reviews see Refs. 10, 11, and 73), and therefore poorly enter the blood-brain barrier and are susceptible to proteolysis. Our rapid, reproducible and sensitive assays should allow to screen for numerous inhibitor candidates. Our data allow to establish that the length of the C-terminal tails adjacent of a stabilizing statine group is a more drastic structural requirement to maintain full efficiency than length of the N-terminal moiety. Our data also showed the potential of designing fully specific BACE1 or BACE2 inhibitors. Thus, we found inhibitors of BACE1 or

BACE2 that appeared inactive on the parent protease. Although these compounds clearly did not fully discriminate between the two enzymes, they constitute the starting point of a rational design leading to the selection of fully selective blocking agents. This is not only a biochemical challenge but indeed an important issue. Thus, BACE2 cleaves mainly inside the Aβ sequence, at a nonamyloidogenic site and therefore likely contributes to the depletion of Aβ or BACE1-generated Aβ-related species. In this case that likely reflects most of sporadic AD, inhibition of BACE1 but not BACE2 is an important issue. On the other hand, BACE2 is encoded by chromosome 21 and therefore possibly contributes to the neuropathological AD-like stigmata that take place in trisomic brains. Thus it has been shown that brains affected with Down's syndrome display elevated BACE2 expression (15). BACE2 activity also appears increased by the Flemish AD mutation (28). In these cases, a BACE2-specific inhibitor would be likely very useful.

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